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(57) Abstract

The present invention relates to a novel I-FLICE-1 or I-FLICE-2 protein which is a novel inhibitor of TNFR-1 and CD-95 induced apoptosis. In particular, isolated nucleic acid molecules are provided encoding the human I-FLICE-1 or I-FLICE-2 protein. I-FLICE-1 or I-FLICE-2 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of I-FLICE-1 or I-FLICE-2 activity. Also provided are therapeutic methods for treating diseases and disorders associated with apoptosis.

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I-FLICE, A NOVEL INHIBITOR OF TUMOR NECROSIS FACTOR RECEPTOR-1 AND CD-95 INDUCED APOPTOSIS

Background of the Invention

Field of the Invention

The present invention relates to a novel inhibitor of TNFR-1 and CD-95 induced apoptosis. More specifically, isolated nucleic acid molecules are provided encoding a human I-FLICE (Inhibitor of FLICE (FADD-like ICE)) polynucleotides. I-FLICE polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of I-FLICE activity. Also provided are therapeutic methods for treating diseases and disorders associated with apoptosis.

Related Art

The cell death machinery is conserved throughout evolution and is composed of activators, inhibitors, and effectors (Chinnaiyan, A.M. and Dixit, V.M., *Curr. Biol. 6*:555-562 (1996)). The effector arm of the cell death pathway is composed of a rapidly growing family of cysteine aspartate-specific proteases termed caspases (Alnemri, E.S., et al., *Cell 87*:171 (1996)). As implied by the name, these cysteine proteases cleave substrates following an aspartate residue (Alnemri, E.S., et al., *Cell 87*:171 (1996); Walker, N.P., et al., *Cell 78*:343-352 (1994)). Caspases are normally present as single polypeptide zymogens and contain an amino-terminal prodomain, and large and small catalytic subunits (Wilson, K.P., et al., *Nature 370*:270-274 (1994); Rotonda, J., et al., *Nat. Struct. Biol. 3*:619-625 (1996); Fraser, A. and Evan, G., *Cell 85*:781-784 (1996)). The two chain active enzyme (composed of the large and small subunits) is obtained following proteolytic processing at internal Asp residues (Wilson, K.P., et al., *Nature 370*:270-274 (1994); Rotonda, J., et al., *Nat. Struct. Biol. 3*:619-625 (1996); Fraser, A. and Evan, G., *Cell 85*:781-784 (1996)). As such, caspases are capable of activating each other in a manner analogous to zymogen activation that

is observed in the coagulation cascade (Boldin, M.P., et al., *Cell 85*:805-815 (1996)). The identification of FLICE and Mch4/FLICE2 as receptor associated caspases suggested a surprisingly direct mechanism for activation of the death pathway by the cytotoxic receptors CD-95 and TNFR-1 (Boldin, M.P., et al., *Cell 85*:805-815 (1996); Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995)). Upon activation, both receptors use their death domains to bind the corresponding domain in the adaptor molecule FADD (Fas-associated death domain protein) (Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995)). Dominant negative versions of FADD that lack the N-terminal segment but still retain the death domain potently inhibit both CD-95 and TNFR-1 induced apoptosis (Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996); Muzio, M., et al., *J. Biol. Chem. 272*:2952-2956 (1997)). Given the importance of the N-terminal segment in engaging the death pathway, it has been termed the death effector domain (DED) (Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996)).

Remarkably, the DED is present within the prodomain of FLICE and Mch4/FLICE2 and mutagenesis studies suggest that a homophilic interaction between the DED of FADD and the corresponding domain in FLICE or Mch4/FLICE2 is responsible for the recruitment of these proteases to the CD-95 and TNFR-1 signalling complexes (Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995); Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996)). Taken together, these data are consistent with FLICE and Mch4/FLICE2 being apical enzymes that initiate precipitous proteolytic processing of downstream caspases resulting in apoptosis (Boldin, M.P., et al., *Cell 85*:805-815 (1996); Srinivasula, S.M., et al., *PNAS 93*:14486-14491 (1996); Fernandes-Alnemri, T., et al., *PNAS 93*:7464-7469 (1996); Henkart, P.A., *Immunity 4*:195-201 (1996)). A number of viral gene products antagonize CD-95

and TNFR-1 mediated killing as a means to persist in the infected host (Shen, Y. and Shenk, T.S., *Current Opinion in Genetics and Development* *5*:105-111 (1995)). The poxvirus encoded serpin CrmA and baculovirus gene product p35 are direct caspase inhibitors (Walker, N.P., et al., *Cell* *78*:343-352 (1994)). In contrast, the molluscum contagiosum virus protein MC159 and the equine herpes virus protein E8 encode DED-containing decoy molecules that bind to either FADD (MC159) or FLICE (E8) and disrupt assembly of the receptor signalling complex, thereby abrogating the death signal (Hu, S., et al., *J. Biol. Chem.* *272*:9621-9624 (1997); Bertin, J., et al., *PNAS* *94*:1172-1176 (1997); Thome, M., et al., *Nature* *386*:527-521 (1997)). The existence of these viral inhibitors has raised the question of whether functionally equivalent molecules are encoded in the mammalian genome.

There is a need for factors, such as the polypeptides of the present invention, that are useful for inhibiting apoptosis for therapeutic purposes, for example, in the treatment of Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage. There is a need, therefore, for the identification and characterization of such factors that are inhibitors of apoptosis, such as the I-FLICE-1 and I-FLICE-2 polypeptides of the present invention, which can play a role in preventing, ameliorating or correcting the diseases and disorders associated with apoptosis.

Summary of the Invention

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the I-FLICE-1 polypeptide having the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence encoded by the cDNA clone deposited in a bacterial host as ATCC Deposit Number 209038 on

May 15, 1997. The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the I-FLICE-2 polypeptide having the amino acid sequence shown in SEQ ID NO:6 or the amino acid sequence encoded by the cDNA clone deposited in a bacterial host as ATCC Deposit Number 209041 on May 15, 1997.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of I-FLICE-1 or I-FLICE-2 polypeptides or peptides by recombinant techniques.

The invention further provides an isolated I-FLICE-1 or I-FLICE-2 polypeptides having an amino acid sequence encoded by the polynucleotides described herein.

The invention further provides a diagnostic method useful during diagnosis or prognosis of a disease states resulting from aberrant cell proliferation due to alterations in I-FLICE-1 or I-FLICE-2 expression.

The present invention also provides a screening method for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular activity of either an I-FLICE-1 or I-FLICE-2 polypeptide. The method involves contacting cells which express one or both of the I-FLICE-1 or I-FLICE-2 polypeptides with a candidate compound, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the polypeptide activity and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the activity.

An additional aspect of the invention is related to a method for treating an individual in need of an increased level of I-FLICE-1 or I-FLICE-2 activity in the body comprising administering to such an individual a composition comprising

a therapeutically effective amount of an isolated I-FLICE-1 or I-FLICE-2 polypeptide of the invention or an agonist thereof.

A still further aspect of the invention is related to a method for treating an individual in need of a decreased level of I-FLICE-1 or I-FLICE-2 activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of an I-FLICE-1 or I-FLICE-2 antagonist.

Brief Description of the Figures

FIG. 1A-1B shows the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of I-FLICE-1 (HSLAZ11). The protein has 480 amino acid residues and a deduced molecular weight of about 55.3 kDa.

FIG. 2 shows the regions of similarity between the amino acid sequences of the I-FLICE-1, I-FLICE-2, FLICE (SEQ ID NO:3), and Mch4 (SEQ ID NO:4). Shading (with solid black) indicates residues that match the consensus sequence exactly.

FIG. 3 shows an analysis of the I-FLICE-1 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index-Jameson-Wolf" graph, amino acid residues about 41 to about 92, about 155 to about 249, about 332 to about 447 in FIG. 1A-1B (SEQ ID NO:2) correspond to the shown highly antigenic regions of the I-FLICE-1 protein.

FIG. 4A-4C shows the nucleotide (SEQ ID NO:5) and deduced amino acid (SEQ ID NO:6) sequences of I-FLICE-2 (HCEBJ50). The protein has 348 amino acid residues and a deduced molecular weight of about 39.2 kDa.

FIG. 5 shows an analysis of the I-FLICE-2 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues about 62 to

about 136, about 184 to about 193, about 205 to about 341 in FIG. 4A-4C (SEQ ID NO:6) correspond to the shown highly antigenic regions of the I-FLICE-2 protein.

FIG. 6A-6B shows I-FLICE-1 inhibition of apoptosis. Overexpression of I-FLICE-1 attenuated TNFR-1 (panel A) and CD-95 (panel B) induced cell death. 293 (panel A) or 293-EBNA (panel B) cells were co-transfected with the indicated plasmids together with the reporter construct pCMV β-galactosidase. The data shown are the percentage of blebbing blue cells as a function of total number of blue cells counted.

Detailed Description

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding an I-FLICE-1 or I-FLICE-2 polypeptide having the amino acid sequence shown in SEQ ID NO:2 or SEQ ID NO:6, respectively, which was determined by sequencing a cloned cDNA. The I-FLICE-1 protein of the present invention shares sequence homology with FLICE and Mch4 (FIG. 2) (SEQ ID NOs:3 and 4). The nucleotide sequence shown in SEQ ID NO:1 was obtained by sequencing a cDNA clone (HSLAZ11), which was deposited on May 15, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given accession number 209038. The deposited clone is inserted in the pBluescript SK(-) plasmid (Stratagene, La Jolla, CA) using the EcoRI and XhoI restriction endonuclease cleavage sites. The I-FLICE-2 protein of the present invention shares sequence homology with FLICE and Mch4 (FIG. 2 (SEQ ID NOs:3 and 4)). The nucleotide sequence shown in SEQ ID NO:5 was obtained by sequencing a cDNA clone (HCEBJ50), which was deposited on May 15, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given accession number 209041. The deposited clone is inserted in the pBluescript SK(-) plasmid (Stratagene, La Jolla, CA) using the EcoRI and XhoI restriction endonuclease cleavage sites.

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Using the information provided herein, such as the nucleotide sequence in SEQ ID NO:1 or SEQ ID NO:5, a nucleic acid molecule of the present invention encoding an I-FLICE-1 or I-FLICE-2 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule described in SEQ ID NO:1 was discovered in a cDNA library derived from human umbilical vein endothelial cell. The gene was also identified in cDNA libraries from smooth muscle. The determined nucleotide sequence of the I-FLICE-1 cDNA of SEQ ID NO:1 contains an open reading frame encoding a protein of about 480 amino acid residues and a deduced

molecular weight of about 55.3 kDa. The I-FLICE-1 protein shown in SEQ ID NO:2 is overall about 29% identical and about 54% similar to FLICE (FIG. 2 (SEQ ID NO:3)).

Also illustrative of the invention, the nucleic acid molecule described in SEQ ID NO:5 was discovered in a cDNA library derived from human umbilical vein endothelial cell. The gene was also identified in cDNA libraries from brain tissue isolated from the cerebellum. The determined nucleotide sequence of the I-FLICE-2 cDNA of SEQ ID NO:5 contains an open reading frame encoding a protein of about 348 amino acid residues and a deduced molecular weight of about 39 kDa. The I-FLICE-2 protein shown in SEQ ID NO:6 is overall about 28% identical and about 54% similar to FLICE (FIG. 2 (SEQ ID NO:3)).

In addition, I-FLICE-1 and I-FLICE-2 are nearly identical over the majority of their sequences; however, I-FLICE-1 has additional amino acids comprising the N-terminal region of the protein. The amino terminal domains of both I-FLICE-1 and I-FLICE-2 exhibit significant sequence similarity to the DED domain of the FADD protein (Hu, S. et al., *J. Biol. Chem. 272*:17255-17257 (1997); Irmler, M., et al., *Nature 388*:190-195 (1997)), the domain through which FLICE proteins and death receptors interact. The amino terminal domain of I-FLICE-2 consists of only a single DED/FADD homology domain (comprising amino acid residues from about 1 to about 75 in SEQ ID NO:6), while the additional amino acids found in the amino terminal domain of I-FLICE-1 appear to provide a second DED/FADD homology domain (comprising amino acid residues from about 1 to about 75 and amino acids residues from about 91 to about 171 in SEQ ID NO:2). The carboxy terminal domains of the both I-FLICE-1 and I-FLICE-2 also contain significant sequence similarity to the active subunit domains of the ICE/CED-3 family of cysteine proteases (amino acids residues from about 172 to about 375 and amino acid residues from about 376 to about 480 in SEQ ID NO:2; amino acids residues from about 76 to about 252 and amino acid residues from about 253 to about 348 in SEQ ID NO:6). Neither I-FLICE-1 or I-FLICE-2 contain the catalytic cysteine that is normally embedded

in the conserved pentapeptide QACRG or QACQG motif present in all known caspases. Rather, both I-FLICE-1 and I-FLICE-2 have the pentapeptide sequence QNYVV (amino acid residues from about 358 to about 362 in SEQ ID NO:2 and amino acid residues from about 244 to about 248 in SEQ ID NO:6). Further, only three of seven conserved residues that form the substrate binding pocket found in all caspases are present in I-FLICE-1 and I-FLICE-2. Given the lack of conservation of key residues involved in catalysis and substrate binding, it can be concluded that I-FLICE-1 and I-FLICE-2 are not cysteine proteases and are incapable of substrate binding, thus, providing these proteins with a dominant negative inhibitory function. I-FLICE-1 and I-FLICE-2 are the first examples of catalytically inert caspases that can inhibit apoptosis.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) shown in SEQ ID NO:1 or EQ ID NO:5; DNA molecules comprising the coding sequence for the I-FLICE -1 or I-FLICE-2 protein; and DNA molecules which comprise a sequence

substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the I-FLICE-1 or I-FLICE-2 protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:1 which have been determined from the following related cDNA clones: HOSBY07R (SEQ ID NO:23), HSAVA13R (SEQ ID NO:24), HLFBD88R (SEQ ID NO:25), HOSAH65R (SEQ ID NO:26), HUVBS23R (SEQ ID NO:27), HHFFJ01RA (SEQ ID NO:28), HUVBL22R (SEQ ID NO:29), and HUVBX15R (SEQ ID NO:30).

The invention also provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:5 (I-FLICE-2) which have been determined from the following related cDNA clones: HTNBE58R (SEQ ID NO:31), HTPBE58R (SEQ ID NO:32), HOSBY07R (SEQ ID NO:23), HSAVA13R (SEQ ID NO:24), HLFBD88R (SEQ ID NO:25), HOSAH65R (SEQ ID NO:26), and HHFFJ01RA (SEQ ID NO:28).

In another aspect, the invention provides isolated nucleic acid molecules encoding the I-FLICE-1 polypeptide having an amino acid sequence as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 209038 on May 15, 1997. The invention also provides isolated nucleic acid molecules encoding the I-FLICE-2 polypeptide having an amino acid sequence as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 209041 on May 15, 1997. In a further embodiment, nucleic acid molecules are provided encoding the I-FLICE-1 or I-FLICE-2 polypeptide or the full-length I-FLICE polypeptide lacking the N-terminal methionine. The invention also provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:5 or the nucleotide sequence of the I-FLICE-1 or I-FLICE-2 cDNA contained in the above-described deposited clones, or a nucleic acid molecule having a sequence complementary to one of the

above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping, by *in situ* hybridization with chromosomes, and for detecting expression of the I-FLICE-1 or I-FLICE-2 gene in human tissue, for instance, by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:5 is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course larger DNA fragments 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, 2000, or 2016 nt in length of the sequence shown in SEQ ID NO:1 are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 209038 or as shown in SEQ ID NO:1. Similarly, larger DNA fragments 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, 2000, 2050, 2100, 2150, 2200, 2250, 2300, 2350, 2400, 2450, 2500, or 2547 nt in length of the sequence shown in SEQ ID NO:5 are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 209041 or as shown in SEQ ID NO:5. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1 or SEQ ID NO:5.

In a more specific embodiment, the nucleic acid molecules of the present invention do not include the sequences, nucleic acid molecules (*e.g.*, clones), or nucleic acid inserts identified in one or more of the following GenBank Accession Reports: AA001257, AA151642, AA149562, C05730, AA565691, AA467756, D83882, AA002262, AA115793, AA467995, AA115792, AA467938, W60406, AA358042, AA468056, W23795, AA358043, T93307, AA453850, AA379905, AA296229, H15978, AA501289, AA296309, AA296174, T30922, T48754, AA453766, C05795, AA198928, N94588, H15052, Z42895, F13176, W52946, AA558404, AA070614, AA613966, AA525331, AA663074, AA135811, AA526099, AA302978, H68343, AA610255, AA229005, T05118, T30864, AA302968, or AA364006, all of which are incorporated herein by reference.

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the I-FLICE-1 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 41 to about 92 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 155 to about 249 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 332 to about 474 in SEQ ID NO:2. The inventors have determined that the above polypeptide fragments are antigenic regions of the I-FLICE-1 protein. Methods for determining other such epitope-bearing portions of the I-FLICE-1 protein are described in detail below.

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the I-FLICE-2 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 62 to about 136 in SEQ ID NO:6; a polypeptide comprising amino acid residues from about 184 to about 193 in SEQ ID NO:6; a polypeptide comprising amino acid residues from about 205 to about 341 in SEQ ID NO:6. The inventors have determined that the above polypeptide fragments are antigenic regions of the

I-FLICE-2 protein. Methods for determining other such epitope-bearing portions of the I-FLICE-2 protein are described in detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit 209038 or ATCC Deposit 209041. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNAs or the nucleotide sequence as shown in SEQ ID NO:1 or SEQ ID NO:5). Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3′ terminal poly(A) tract of the I-FLICE-1 cDNA shown in SEQ ID NO:1 or the I-FLICE-2 cDNA shown in SEQ ID NO:5), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode an I-FLICE-1 or I-FLICE-2 polypeptide may include, but are not limited to those encoding the amino acid sequence of the polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5′ and 3′ sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*:767-778 (1984). As discussed below, other such fusion proteins include the I-FLICE-1 or I-FLICE-2 fused to Fc at the N- or C-terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the I-FLICE-1 or I-FLICE-2 protein. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism.

Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions, which may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the I-FLICE-1 or I-FLICE-2 protein or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209038; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:6; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:6, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209041; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Additional embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least

95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 75 in SEQ ID NO:2; (b) a nucleotide sequence encoding a polypeptide comprising amino acids from about 91 to about 171 in SEQ ID NO:2; (c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 172 to about 375 in SEQ ID NO:2; (d) a nucleotide sequence encoding a polypeptide comprising amino acids from about 376 to about 480 in SEQ ID NO:2; (e) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 75 in SEQ ID NO:6; (f) a nucleotide sequence encoding a polypeptide comprising amino acids from about 76 to about 252 in SEQ ID NO:6; (g) a nucleotide sequence encoding a polypeptide comprising amino acids from about 253 to about 348 in SEQ ID NO:6; (h) or a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), or (g).

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding an I-FLICE polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding an I-FLICE polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5′ or 3′ terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide

sequence shown in SEQ ID NO:1 or SEQ ID NO:5 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:5 or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having I-FLICE activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having I-FLICE activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having I-FLICE activity include, *inter alia,* (1) isolating the I-FLICE-1 or I-FLICE-2 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the I-FLICE-1 or I-FLICE-2 gene, as described in Verma *et al., Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting I-FLICE-1 or I-FLICE-2 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:5 or to a nucleic acid sequence of the deposited cDNA which do, in fact, encode a polypeptide having I-FLICE protein activity. By "a polypeptide having I-FLICE activity" is intended polypeptides exhibiting I-FLICE-1 or I-FLICE-2 activity in a particular biological assay. For example, I-FLICE-1 or I-FLICE-2 protein activity can be measured using the cell death assay as described in Example 6.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence of the deposited cDNA or a nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:5 will encode "a polypeptide having I-FLICE activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having I-FLICE activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.,* "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Vectors and Host Cells

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of I-FLICE-1 or I-FLICE-2 polypeptides or fragments thereof by recombinant techniques.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac, trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods In Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other

hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5-receptor has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

The I-FLICE-1 or I-FLICE-2 protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

I-FLICE-1 and I-FLICE-2 Polypeptides and Fragments

The invention further provides an isolated I-FLICE-1 or I-FLICE-2 polypeptide having the amino acid sequence encoded by the deposited cDNAs,

or the amino acid sequence in SEQ ID NO:2 or SEQ ID NO:6, or a peptide or polypeptide comprising a portion of the above polypeptides.

It will be recognized in the art that some amino acid sequences of the I-FLICE-1 or I-FLICE-2 polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the I-FLICE-1 or I-FLICE-2 polypeptide which show substantial I-FLICE-1 or I-FLICE-2 polypeptide activity or which include regions of I-FLICE-1 or I-FLICE-2 protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990).

Thus, the fragment, derivative or analog of the polypeptide of SEQ ID NO:2 or SEQ ID NO:6, or that encoded by the deposited cDNAs, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the I-FLICE-1 or I-FLICE-2 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al., Clin. Exp. Immunol. 2*:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al., Nature 361*:266-268 (1993) describes certain mutations resulting in selective binding of TNF-α to only one of the two known types of TNF receptors. Thus, the I-FLICE-1 or I-FLICE-2 of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above and below. Generally speaking, the number of substitutions for any given I-FLICE-1 or I-FLICE-2 polypeptide, or mutant thereof, will not be more than 50, 40, 30, 20, 10, 5, or 3, depending on the objective.

Amino acids in the I-FLICE-1 or I-FLICE-2 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. Sites that are critical for ligand interactions can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell. For example, a recombinantly produced version of the I-FLICE-1 or I-FLICE-2 polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67:*31-40 (1988).

The polypeptides of the present invention include the polypeptide encoded by the deposited cDNA; a polypeptide comprising amino acids about 1 to about 480 in SEQ ID NO:2; a polypeptide comprising amino acids about 2 to about 480 in SEQ ID NO:2; as well as polypeptides which are at least 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to those described above and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA; a polypeptide comprising amino acids about 1 to about 348 in SEQ ID NO:6; a polypeptide comprising amino acids about 2 to about 348 in SEQ ID NO:6; as well as polypeptides which are at least 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to those described above and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

The polypeptides of the present invention further include the polypeptide comprising amino acids from about 1 to about 75 in SEQ ID NO:2; amino acids from about 91 to about 171 in SEQ ID NO:2; amino acids from about 172 to about 375 in SEQ ID NO:2; amino acids from about 376 to about 480 in SEQ ID NO:2; amino acids from about 1 to about 75 in SEQ ID NO:6; amino acids from about 76 to about 252 in SEQ ID NO:6; amino acids from about 253 to about 348 in SEQ ID NO:6; as well as polypeptides which are at least 95% identical, still

more preferably at least 96%, 97%, 98% or 99% identical to those described above and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of an I-FLICE-1 or I-FLICE-2 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the I-FLICE-1 or I-FLICE-2 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or SEQ ID NO:6 or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in

homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The polypeptide of the present invention are useful as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide described herein. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al., Proc. Natl. Acad. Sci. USA 81*:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science 219:*660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al., Cell 37*:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate I-FLICE-1 -specific antibodies include: a polypeptide comprising amino acid residues from about 41 to about 92 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 155 to about 249 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 332 to about 474 in SEQ ID NO:2. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the I-FLICE-1 protein.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate I-FLICE-2 -specific antibodies include: a polypeptide comprising amino acid residues from about 62 to about 136 in SEQ ID NO:6; a polypeptide comprising amino acid residues from about 184 to about 193 in SEQ ID NO:6; a polypeptide comprising amino acid residues from about 205 to about 341 in SEQ ID NO:6. The inventors have determined that the above polypeptide fragments are antigenic regions of the I-FLICE-2 protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R. A. (1985) General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA 82*:5131-5135. This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

As one of skill in the art will appreciate, I-FLICE-1 or I-FLICE-2 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has

been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al., Nature 331*:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric I-FLICE-1 or I-FLICE-2 protein or protein fragment alone (Fountoulakis *et al., J. Biochem 270*:3958-3964 (1995)).

Disease Diagnosis and Prognosis

It is believed that certain tissues in mammals with specific disease states associated with aberrant cell survival express significantly altered levels of I-FLICE-1 or I-FLICE-2 and mRNA encoding I-FLICE-1 or I-FLICE-2 when compared to a corresponding "standard" mammal, i.e., a mammal of the same species not having the disease state. Thus, the present invention is useful for detecting such states in mammals. Preferred mammals include monkeys, apes, cats, dogs, cows, pigs, horses, rabbits and humans. Particularly preferred are humans.

It is further believed that enhanced levels of I-FLICE-1 or I-FLICE-2 can be detected in certain body fluids (e.g., sera, plasma, urine, and spinal fluid) from mammals with the disease state when compared to analogous fluids from mammals of the same species not having the disease state. Thus, the invention provides a diagnostic method useful during diagnosis of disease states, which involves assaying the expression level of the gene encoding I-FLICE-1 or I-FLICE-2 in mammalian cells or body fluid and comparing the gene expression level with a standard I-FLICE-1 or I-FLICE-2, whereby an increase or decrease in the gene expression level over the standard is indicative of certain disease states associated with aberrant cell survival.

Where diagnosis of a disease state involving I-FLICE-1 or I-FLICE-2 of the present invention has already been made according to conventional methods, the present invention is useful as a prognostic indicator, whereby patients exhibiting significantly aberrant I-FLICE-1 or I-FLICE-2 gene expression levels will experience a worse clinical outcome relative to patients expressing the gene at a lower level.

By "assaying the expression level of the gene encoding I-FLICE-1 or I-FLICE-2" is intended qualitatively or quantitatively measuring or estimating the level of I-FLICE-1 or I-FLICE-2 protein or the level of the mRNA encoding I-FLICE-1 or I-FLICE-2 protein in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the I-FLICE-1 or I-FLICE-2 protein level or mRNA level in a second biological sample).

Preferably, the I-FLICE-1 or I-FLICE-2 protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard I-FLICE-1 or I-FLICE-2 protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disease state. As will be appreciated in the art, once a standard I-FLICE-1 or I-FLICE-2 protein level or mRNA level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source which contains I-FLICE-1 or I-FLICE-2 protein or mRNA. Biological samples include mammalian body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain I-FLICE-1 or I-FLICE-2 protein, and ovarian, prostate, heart, placenta, pancreas liver, spleen, lung, breast, umbilical tissue, and other tissues. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, hormone-dependent tumors, and cancers of the breast, ovary, prostate, bone, liver, lung, pancreas, and spleen); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with decreased cell survival, or increased apoptosis, include Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage.

Assays available to detect levels of proteins are well known to those of skill in the art, for example, radioimmunoassays, competitive-binding assays, Western blot analysis, and preferably an ELISA assay may be employed.

I-FLICE-1 or I-FLICE-2 specific antibodies can be raised against the intact I-FLICE-1 or I-FLICE-2 protein or an antigenic polypeptide fragment thereof, which may presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (mAb) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of specifically binding to the I-FLICE-1 or I-FLICE-2 protein. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)). Thus, these fragments are preferred.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the I-FLICE-1 or I-FLICE-2

protein or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of I-FLICE-1 or I-FLICE-2 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or I-FLICE-1 or I-FLICE-2 protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al., Nature 256*:495 (1975); Kohler *et al., Eur. J. Immunol. 6*:511 (1976); Kohler *et al., Eur. J. Immunol. 6*:292 (1976); Hammerling *et al.*, In: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., (1981) pp. 563-681).

Assaying I-FLICE-1 or I-FLICE-2 protein levels in a biological sample can occur using antibody-based techniques. For example, I-FLICE-1 or I-FLICE-2 protein expression in tissues can be studied with classical immunohistological methods (Jalkanen, M., *et al., J. Cell. Biol. 101*:976-985 (1985); Jalkanen, M., *et al., J. Cell. Biol. 105*:3087-3096 (1987)).

As noted above, other antibody-based methods useful for detecting I-FLICE-1 or I-FLICE-2 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as, Glucose oxidase, and radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Total cellular RNA can be isolated from a biological sample using the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski and Sacchi, *Anal. Biochem. 162*:156-159 (1987). Levels of mRNA encoding the I-FLICE-1 or I-FLICE-2 protein are then assayed using any

appropriate method. These include Northern blot analysis (Harada *et al., Cell 63:*303-312 (1990)), S1 nuclease mapping (Fujita *et al., Cell 49:*357- 367 (1987)), the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR) (Makino *et al., Technique 2:*295-301 (1990)), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Agonists and Antagonists - Assays and Molecules

The invention also provides a method of screening compounds to identify agonists and antagonists of I-FLICE-1 or I-FLICE-2. By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing one or more activity mediated by I-FLICE-1 or I-FLICE-2 polypeptides. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting one or more activity mediated by I-FLICE-1 or I-FLICE-2 polypeptides.

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular activity of either an I-FLICE-1 or I-FLICE-2 polypeptide. The method involves contacting cells which express one or both of the I-FLICE-1 or I-FLICE-2 polypeptides with a candidate compound, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the polypeptide activity and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the activity. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response in the presence of a candidate compound and either an I-FLICE-1 or I-FLICE-2 polypeptide (e.g., decreased or increased TNFR-1 or CD-95 induced apoptosis, binding of I-FLICE-1 or I-FLICE-2 to natural cellular ligands such as FLICE and Mch4/FLICE2).

Potential antagonists include small organic molecules amino acid sequences which bind to I-FLICE-1 or I-FLICE-2, fragments of I-FLICE-1 and I-FLICE-2, as well as anti-I-FLICE-1 and anti-I-FLICE-2 antibodies. Fragments of I-FLICE-1 and I-FLICE-2, which may be naturally occurring or synthetic, antagonize I-FLICE-1 and I-FLICE-2 polypeptide mediated activities by competing for binding to natural cellular ligands. Small organic molecules can antagonize I-FLICE-1 and I-FLICE-2 polypeptide mediated activities by binding either competitively or non-competitively to I-FLICE-1 or I-FLICE-2 or a cellular ligand of these proteins. Examples of small molecules include but are not limited to nucleotide sequences and small peptides or peptide-like molecules. Such molecules may be produced and screened for activity by a variety of methods (e.g., Light and Lerner, *Bioorganic & Medicinal Chemistry 3(7)*:955-967 (1995); Cheng *et al.*, *Gene 171*:1-8 (1996); Gates *et al.*, *J. Mol. Biol. 255*:373-386 (1996)).

Similarly, potential agonists also include fragments of the polypeptides of the present invention, as well as anti-I-FLICE-1 and anti-I-FLICE-2 antibodies. Fragments of these proteins can act as agonists of I-FLICE-1 and I-FLICE-2 polypeptide mediated activities by binding to natural cellular ligands and inducing activities associated with the full-length protein. Agonists and antagonists of the present invention also include amino acid sequences having 95% or more identity to those shown in SEQ ID NOs:2 and 6, or fragments thereof.

Other potential antagonists include antisense oligonucleotides and oligonucleotides capable of forming triple helices with the sequences shown in SEQ ID NOs:1 and 5. Once a gene sequence is known, antisense and triple helix technologies can be used to regulate gene expression. Okano, *J. Neurochem. 56*:560 (1991); OLIGONUCLEOTIDES AS INHIBITORS OF GENE EXPRESSION, CRC Press, Boca Raton, FL. (1988); Dervan *et al., Science 251*:1360 (1991); Cooney *et al., Science 241*:456 (1988); Lee *et al., Nucl. Acids Res. 6*:3073 (1979). In regards to antisense technology, for example, an oligonucleotide may be designed which is complementary to a portion of the I-FLICE-1 or I-FLICE-2 DNA

sequences which is transcribed into RNA. This oligonucleotide may be delivered to cells in a number of forms, including as antisense RNA or incorporated into an expression vector. If incorporated into an expression vector, the oligonucleotide is generally orientated in a manner that an RNA molecule is produced upon *in vivo* expression which is complementary to that of the I-FLICE-1 or I-FLICE-2 mRNA sequence. The expressed antisense RNA molecule will hybridize to I-FLICE-1 or I-FLICE-2 mRNA and block translation *in vivo*.

The experiments set forth in Example 5 demonstrate that I-FLICE-1 binds to both FLICE and Mch4/FLICE2. Immunprecipitation assays similar to that described in Example 5 can be used to identify additional molecules which bind to I-FLICE-1 and I-FLICE-2. Such binding molecules are candidate antagonists and agonists.

Example 6 sets forth a cell death assay used to demonstrate that overexpression of I-FLICE-1 results in the inhibition of TNFR-1 and CD-95 induced cell death. This assay can also be used to screen for compounds having agonistic and antagonistic activity directed to I-FLICE-1 and I-FLICE-2. Such a screening method is used to determine whether the compound increases or decreases TNFR-1 and CD-95 induced cell death in the presence of I-FLICE-1 or I-FLICE-2 either individually or in combination.

Proteins and other compounds which bind the I-FLICE-1 or I-FLICE-2 polypeptide domains are also candidate agonists and antagonists according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature 340*:245-246 (1989); Gyuris *et al., Cell 75*:791-803 (1993); Zervos *et al., Cell 72*:223 -232 (1993)).

The agonists may be employed for instance to enhance the action of I-FLICE-1 or I-FLICE-2 polypeptides, for example, in the treatment of Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of

endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage.

The antagonists may be employed for instance to inhibit the action of I-FLICE-1 or I-FLICE-2 polypeptides, for example, in the treatment of cancers (such as follicular lymphomas, carcinomas with p53 mutations, hormone-dependent tumors, and cancers of the breast, ovary, prostate, bone, liver, lung, pancreas, and spleen); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection.

The agonists and antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

Therapeutics

The novel mammalian inhibitors designated I-FLICE-1 and I-FLICE-2 (for inhibitor of FLICE) of the present invention, are catalytically inactive structural homologues of FLICE and Mch4/FLICE2 that inhibit both TNFR-1 and CD-95 induced apoptosis. These are the first examples of a naturally occurring catalytically inactive caspase that can act as a dominant negative inhibitor of apoptosis. The polypeptides of the present invention are useful for inhibiting apoptosis for therapeutic purposes, for example, in the treatment of Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage.

Modes of Administration

It will be appreciated that conditions caused by a decrease in the standard or normal level of I-FLICE-1 or I-FLICE-2 activity in an individual, can be treated by administration of I-FLICE-1 or I-FLICE-2 protein. Thus, the invention further provides a method of treating an individual in need of an increased level of I-FLICE-1 or I-FLICE-2 activity comprising administering to such an individual a pharmaceutical composition comprising an effective amount of an isolated I-FLICE-1 or I-FLICE-2 polypeptide of the invention, particularly a mature form of the I-FLICE-1 or I-FLICE-2, effective to increase the I-FLICE-1 or I-FLICE-2 activity level in such an individual.

As a general proposition, the total pharmaceutically effective amount of I-FLICE-1 or I-FLICE-2 polypeptide administered parenterally per dose will be in the range of about 1 μg/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the I-FLICE-1 or I-FLICE-2 polypeptide is typically administered at a dose rate of about 1 μg/kg/hour to about 50 μg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Pharmaceutical compositions containing the I-FLICE-1 or I-FLICE-2 of the invention may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), bucally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Chromosome Assays

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of an I-FLICE-1 or I-FLICE-2 protein gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, in some cases, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3′ untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 bp. For a review of this technique, see Verma *et al., Human Chromosomes: A Manual Of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance In Man*, available on-line through Johns Hopkins University, Welch

Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Examples

Example 1(a): Expression and Purification of I-FLICE-1 in E. coli

The bacterial expression vector pQE60 is used for bacterial expression in this example. (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide.

The DNA sequence encoding the desired portion I-FLICE-1 protein is amplified from the deposited cDNA clone using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portion of the I-FLICE-1 protein and to sequences in the deposited construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the protein, the 5' primer has the sequence: 5' CGCCCATGGCTGAAGTCATCCATCAG 3' (SEQ ID NO:7) containing the underlined NcoI restriction site followed by 16 (i.e., 275-291) nucleotides complementary to the amino terminal coding sequence of the I-FLICE-1 sequence in FIG. 1A-1B (SEQ ID NO:1). One of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a DNA segment encoding any desired portion of the complete protein in a shorter or longer form. The 3' primer has the sequence: 5' CGCAAGCTTGTGCTGGGATTACAGGTG 3' (SEQ ID NO:8) containing the underlined HindIII restriction site followed by 18 (i.e., 1740-1758) nucleotides complementary to the 3' end of the coding sequence immediately before the stop codon in the I-FLICE-1 DNA sequence in FIG. 1A-1B (SEQ ID NO:1), with the coding sequence aligned with the restriction site so as to maintain its reading frame with that of the six His codons in the pQE60 vector.

The amplified I-FLICE-1 DNA fragment and the vector pQE60 are digested with NcoI/HindIII and the digested DNAs are then ligated together. Insertion of the I-FLICE-1 DNA into the restricted pQE60 vector places the I-FLICE-1 protein coding region downstream from the IPTG-inducible promoter and in-frame with an initiating AUG and the six histidine codons.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing I-FLICE-1 protein, is available commercially from QIAGEN, Inc., *supra.* Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant

colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. Isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the I-FLICE-1 is loaded onto a nickel-nitrilo-tri-acetic acid ("NiNTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the NI-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist, 1995, QIAGEN, Inc., *supra).* Briefly the supernatant is loaded onto the column in 6 M guanidine-HCl, pH8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH8, then washed with 10 volumes of 6 M guanidine-HCl pH6, and finally the I-FLICE-1 is eluted with 6 M guanidine-HCl, pH5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM imidazole. Imidazole is removed by a final dialyzing step

against PBS or 50 mM sodium acetate pH6 buffer plus 200 mM NaCl. The purified protein is stored at 4°C or frozen at -80°C.

Example 1(b): Expression and Purification of I-FLICE-2 in E. coli

The bacterial expression vector pQE60 is used for bacterial expression in this example. (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide.

The DNA sequence encoding the desired portion I-FLICE-2 protein is amplified from the deposited cDNA clone using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portion of the I-FLICE-2 protein and to sequences in the deposited construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the protein, the 5' primer has the sequence: 5' CGC<u>CCATGG</u>AGATTGGTGAGGATTTG 3' (SEQ ID NO:9) containing the underlined NcoI restriction site followed by 17 (i.e., 311-328) nucleotides complementary to the amino terminal coding sequence of the I-FLICE-2 sequence in FIG. 4A-4C (SEQ ID NO:5). one of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a DNA segment encoding any desired portion of the complete protein in a shorter or longer form. The 3' primer has the sequence:

5' CGCAAGCTTAGAGCATGCAGTGTCAG 3' (SEQ ID NO:10) containing the underlined HindIII restriction site followed by 16 (i.e., 1400-1416) nucleotides complementary to the 3' end of the coding sequence immediately before the stop codon in the I-FLICE-2 DNA sequence in FIG. 4A-4C (SEQ ID NO:5), with the coding sequence aligned with the restriction site so as to maintain its reading frame with that of the six His codons in the pQE60 vector.

The amplified I-FLICE-2 DNA fragment and the vector pQE60 are digested with NcoI/HindIII and the digested DNAs are then ligated together. Insertion of the I-FLICE-2 DNA into the restricted pQE60 vector places the I-FLICE-2 protein coding region downstream from the IPTG-inducible promoter and in-frame with an initiating AUG and the six histidine codons.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing I-FLICE-2 protein, is available commercially from QIAGEN, Inc., *supra.* Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. Isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating

the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH8. The cell debris is removed by centrifugation, and the supernatant containing the I-FLICE-2 is loaded onto a nickel-nitrilo-tri-acetic acid ("NiNTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the NI-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist, 1995, QIAGEN, Inc., *supra).* Briefly the supernatant is loaded onto the column in 6 M guanidine-HCl, pH8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH8, then washed with 10 volumes of 6 M guanidine-HCl pH6, and finally the I-FLICE-2 is eluted with 6 M guanidine-HCl, pH5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM imidazole. Imidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH6 buffer plus 200 mM NaCl. The purified protein is stored at 4°C or frozen at -80°C.

Example 2(a): Cloning and Expression of I-FLICE-1 protein in a Baculovirus Expression System

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding the complete protein into a baculovirus to express the I-FLICE-1 protein, using standard methods as described in Summers *et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures,* Texas Agricultural Experimental Station Bulletin No. 1555 (1987).

This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as BamHI and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al., Virology 170*:31-39.

The cDNA sequence encoding the full length I-FLICE-1 protein in the deposited clone, including the AUG initiation codon shown in FIG. 1A-1B (SEQ ID NO:1), is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence:

5' CGC<u>GGATCC</u>GCCATCATGTCTGCTGAAGTCATC 3' (SEQ ID NO:11) containing the underlined BamHI restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (i.e., 268-285) bases of the sequence of the complete I-FLICE-1 protein shown in FIG. 1A-1B, beginning with the AUG initiation codon. The 3' primer has the sequence:

5' CGC<u>GGTACC</u>GTGCTGGGATTACAGGTG 3' (SEQ ID NO:12) containing the underlined, Asp718 restriction site followed by 18 (1740-1758) nucleotides complementary to the 3' noncoding sequence in FIG. 1A-1B (SEQ ID NO:1).

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated herein "F1".

The plasmid is digested with the restriction enzymes BamHI and Asp718 and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V1".

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human I-FLICE-1 gene using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing the I-FLICE-1 gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac I-FLICE-1.

Five μg of the plasmid pBac I-FLICE-1 is co-transfected with 1.0 μg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). 1 μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBac I-FLICE-1 are mixed in a sterile well of a microtiter plate containing 50 μl of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 μl Lipofectin plus 90 μl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix

the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra.* An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V- I-FLICE-1.

To verify the expression of the I-FLICE-1 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat inactivated FBS. The cells are infected with the recombinant baculovirus V- I-FLICE-1 at a multiplicity of infection ("MOI") of about 2. Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). If radiolabeled proteins are desired, 42 hours later, 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal

sequence of the mature protein and thus the cleavage point and length of the secretory signal peptide.

Example 2(b): Cloning and Expression of I-FLICE-2 protein in a Baculovirus Expression System

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding the complete protein into a baculovirus to express the I-FLICE-2 protein, using standard methods as described in Summers *et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures,* Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as BamHI and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al., Virology 170*:31-39.

The cDNA sequence encoding the full length I-FLICE-2 protein in the deposited clone, including the AUG initiation codon shown in FIG. 4A-4C (SEQ ID NO:6) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence:

5' CGCGGATCCGCCATCATGGCAGAGATTGGTGAG 3' (SEQ ID NO:13) containing the underlined BamHI restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (304-321) bases of the sequence of the complete I-FLICE-2 protein shown in FIG. 4A-4C, beginning with the AUG initiation codon. The 3' primer has the sequence:

5' CGCGGTACCAGAGCATGCAGTGTCAG 3' (SEQ ID NO:14) containing the underlined, Asp718 restriction site followed by (i.e., 1400-1416) nucleotides complementary to the 3' noncoding sequence in FIG. 4A-4C (SEQ ID NO:5).

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated herein "F1".

The plasmid is digested with the restriction enzymes BamHI and Asp718 and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V1".

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human I-FLICE-2 gene using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing the I-FLICE-2 gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac I-FLICE-2.

Five μg of the plasmid pBac I-FLICE-2 is co-transfected with 1.0 μg of a commercially available linearized baculovirus DNA ("BaculoGold™

baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). 1 μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBac I-FLICE-2 are mixed in a sterile well of a microtiter plate containing 50 μl of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 μl Lipofectin plus 90 μl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra.* An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 μl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V-I-FLICE-2.

To verify the expression of the I-FLICE-2 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat inactivated FBS. The cells are infected with the recombinant baculovirus V-I-FLICE-2 at a multiplicity of infection ("MOI") of about 2. Six hours later the medium is removed and is

replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). If radiolabeled proteins are desired, 42 hours later, 5 μCi of ^{35}S-methionine and 5 μCi ^{35}S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature protein and thus the cleavage point and length of the secretory signal peptide.

Example 3: Cloning and Expression of I-FLICE in Mammalian Cells

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human HeLa 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV 1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable

marker such as dhfr, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy *et al., Biochem J. 227*:277-279 (1991); Bebbington *et al., Bio/Technology 10:*169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molec. Cell. Biol. 5*:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart *et al., Cell 41*:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 3(a): Cloning and Expression of I-FLICE-1 in COS Cells

The expression plasmid, p I-FLICE-1 HA, is made by cloning a cDNA encoding I-FLICE-1 into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a

hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al., Cell 37:*767-778 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the I-FLICE-1 is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The I-FLICE-1 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of I-FLICE-1 in *E. coli.* Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined SmaI site, a Kozak sequence, an AUG start codon and 17 bases of the 5' coding region of the complete I-FLICE-1 has the following sequence:
5' CGCCCCGGGGCCATCATGTCTGCTGAAGTCATC (268-285) 3' (SEQ ID NO:15). The 3' primer, containing the underlined XbaI site, a stop codon, and 18 bp of 3' coding sequence has the following sequence (at the 3' end):
5' CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAGTGC TGGGATTACAGGTG (1740-1758) 3' (SEQ ID NO:16).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with SmaI and XbaI and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from

resistant colonies and examined by restriction analysis or other means for the presence of the I-FLICE-1-encoding fragment.

For expression of recombinant I-FLICE-1, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook *et al., Molecular Cloning: a Laboratory Manual,* Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of I-FLICE-1 by the vector.

Expression of the I-FLICE-1-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow *et al., Antibodies: A Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson *et al.* cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(b): Cloning and Expression of I-FLICE-1 in CHO Cells

The vector pC4 is used for the expression of I-FLICE-1 protein. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt,

F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J Biol. Chem. 253*:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta, 1097*:107-143, Page, M. J. and Sydenham, M.A. 1991, *Biotechnology 9*:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molec. Cell. Biol. 5*:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al., Cell 41*:521-530 (1985)). Downstream of the promoter are BamHI, XbaI, and Asp718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human β-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the I-FLICE-1 in a regulated way in mammalian cells (Gossen, M., & Bujard, H. 1992, *Proc. Natl. Acad. Sci. USA 89*: 5547-5551). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes BamHI and Asp718 and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete I-FLICE-1 protein including its leader sequence is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence: 5' CGCGGATCCGCCATCATGTCTGCTGAAGTCATC 3' (SEQ ID NO:17) containing the underlined BamHI restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (i.e., 268-285) bases of the sequence of the complete I-FLICE-1 protein shown in FIG. 1A-1B, beginning with the AUG initiation codon. The 3' primer has the sequence: 5' CGCGGTACCGTGCTGGGATTACAGGTG 3' (SEQ ID NO:18) containing the underlined, Asp718 restriction site followed by 18 (1740-1758) nucleotides complementary to the 3' noncoding sequence in FIG. 1A-1B (SEQ ID NO:1).

The amplified fragment is digested with the endonucleases BamHI and Asp718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μg of the expression plasmid pC4 is cotransfected with 0.5 μg of the plasmid pSV2-neo using lipofectin (Felgner *et al., supra).* The plasmid pSV2neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days single clones are

trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 μM, 20 μM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reverse phase HPLC analysis.

Example 3(c): Cloning and Expression of I-FLICE-2 in COS Cells

The expression plasmid, pI-FLICE-2HA, is made by cloning a cDNA encoding I-FLICE-2 into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al., Cell 37:*767-778 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the I-FLICE-2 is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the

CMV promoter. The plasmid construction strategy is as follows. The I-FLICE-2 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of I-FLICE-2 in *E. coli.* Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined BamHI site, a Kozak sequence, an AUG start codon and 17 codons of the 5' coding region of the complete I-FLICE-2 has the following sequence:

5' CGCGGATCCGCCATCATGGCAGAGATTGGTGAG 3' (SEQ ID NO:19).

The 3' primer, containing the underlined XbaI site, a stop codon, and 16 bp of 3' coding sequence has the following sequence (at the 3' end):

5' CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAAGA GCATGCAGTGTCAG 3' (SEQ ID NO:20).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with BamHI and XbaI and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the - transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the I-FLICE-2-encoding fragment.

For expression of recombinant I-FLICE-2, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook *et al., Molecular Cloning: a Laboratory Manual,* Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of I-FLICE-2 by the vector.

Expression of the I-FLICE-2-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow *et al., Antibodies: A Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-

cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson *et al.* cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(d): Cloning and Expression of I-FLICE-2 in CHO Cells

The vector pC4 is used for the expression of I-FLICE-2 protein. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J Biol. Chem. 253*:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta, 1097*:107-143, Page, M. J. and Sydenham, M.A. 1991, *Biotechnology 9*:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molec. Cell. Biol. 5*:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al., Cell 41*:521-530 (1985)). Downstream of the promoter are BamHI, XbaI, and Asp718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human β-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the I-FLICE-2 in a regulated way in mammalian cells (Gossen, M., & Bujard, H. 1992, *Proc. Natl. Acad. Sci. USA 89*:5547-5551). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes BamHI/Asp718 and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete I-FLICE-2 protein sequence is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence:

5' CGC<u>GGATCC</u>GCCATCATGGCAGAGATTGGTGAG 3' (SEQ ID NO:21)

containing the underlined BamHI restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (304-321) bases of the sequence of the

complete I-FLICE-2 protein shown in FIG. 4A-4C, beginning with the AUG initiation codon. The 3' primer has the sequence:
5' CGCGGTACCAGAGCATGCAGTGTCAG 3' (SEQ ID NO:22) containing the underlined, Asp718 restriction site followed by (i.e., 1400-1416) nucleotides complementary to the 3' noncoding sequence in FIG. 4A-4C (SEQ ID NO:5).

The amplified fragment is digested with the endonucleases BamHI and Asp718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μg of the expression plasmid pC4 is cotransfected with 0.5 μg of the plasmid pSV2-neo using lipofectin (Felgner *et al., supra).* The plasmid pSV2neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 μM, 20 μM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reverse phase HPLC analysis.

Example 4(a): Tissue distribution of I-FLICE-1 mRNA expression

Northern blot analysis was carried out to examine I-FLICE-1 gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the I-FLICE-1 protein (SEQ ID NO:1) was labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe was purified using a CHROMA SPIN- 100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe was then used to examine various human tissues for I-FLICE-1 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) were obtained from Clontech and were examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots were mounted and exposed to film at -70°C overnight, and films developed according to standard procedures.

Two transcripts were observed (7.5 kb and 6 kb) which presumably represent mRNA sequences encoding I-FLICE-1 and I-FLICE-2. I-FLICE expression was identified in most tissues and cell lines examined except for the brain and the lymphoblastic leukemia line MOLT4. In particular, I-FLICE expression was evident in peripheral blood leukocytes, spleen, placenta and heart.

Example 4(b): Tissue distribution of I-FLICE-2 mRNA expression

Northern blot analysis is carried out to examine I-FLICE-2 gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the I-FLICE-2 protein (SEQ ID NO:6) is labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to

manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN- 100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for I-FLICE-2 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures.

Example 5: I-FLICE-1 Associates with FLICE and Mch4/FLICE-2

Previous studies have shown that the DED domain is a protein interaction motif that mediates the binding of the adaptor molecule FADD to the effector proteases FLICE and Mch4/FLICE2 (Muzio *et al., Cell 85*:817-27 (1996); Chinnaiyan *et al., Cell 81*:505-12 (1995)). Given the striking structural similarity, the following experiment was performed to determine whether I-FLICE-1 interacted with either FADD or other FLICE-like caspases.

Materials and Methods

Cell Lines and Expression Vectors - Human embryonic kidney 293, 293T and 293-EBNA cells were cultured to Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Expression constructs were made in pcDNA3 or pcDNA3.1/MycHisA (Invitrogen) using standard recombinant methodologies (Sambrook, J. *et al., Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press).

Cloning of I-FLICE-1 - cDNAs corresponding to the partial open reading frame of I-FLICE-1 were identified as sequences homologous to FLICE and Mch4/FLICE2 on searching the Human Genome Sciences data base using established EST methods (Adams, M.D. *et al.*, *Science 252*:1651-1656 (1991) and Adams, M.D. *et al.*, *Nature 355*:632-634 (1992)). Full length cDNAs were obtained by screening a random-primed human umbilical vein endothelial cell cDNA library constructed in the pcDNA1 vector (Invitrogen). The sequence of I-FLICE-1 was confirmed by sequencing plasmid DNA template on both strands by the dideoxy chain termination method employing modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp.).

Transfection, Coimmunoprecipitation and Western Analysis - Transient transfections of 293T cells were performed as described previously (O'Rourke *et al.*, *J. Biol. Chem. 267*:24921-24924 (1992)). Cells were harvested 40 hour following transfection, immunoprecipitation with α-FLAG or αmyc antibodies and analyzed by immunoblotting.

Results and Discussion

Sequence analysis of a full length cDNA revealed a 1443-base pair open reading frame that encoded a novel protein with a predicated molecular mass of 55.3 kDa (FIG. 1A-1B). Given that the protein had striking homology to FLICE and Mch4/FLICE2 but lacked an active site, making it a potential dominant negative inhibitor, it was designated I-FLICE (for inhibitor of FLICE).

The architecture of I-FLICE-1 was strikingly similar to that of FLICE and Mch4/FLICE2, including two N-terminal DED-like tandem repeats and a region that resembled the caspase catalytic domain. Importantly, I-FLICE-1 did not contain the catalytic cysteine that is normally embedded in the conserved pentapeptide QACRG or QACQG motif present in all known caspases. Rather, the pentapeptide sequence was QNYVV. In addition, based on the x-ray crystal structure of caspase-1 (and caspase-3), amino acid residues His^{237} (His^{121}), Gly^{238}

(Gly^{122}), and Cys^{285} (Cys^{163}) are involved in catalysis, while residues Arg^{179} (Arg^{64}), Gin^{283} (Gin^{161}), Arg^{341} (Arg^{207}), and Ser^{347} (Ser^{213}) form a binding pocket for the carboxylate side chain of the P1 aspartic acid (Wilson, K.P. *et al., Nature 370*:270-274 (1994), Rotonda, J. *et al., Nat. Struct. Biol. 3*:619-625 (1996), and Fraser, A. *et al., Cell 85*:781-784 (1996)). These seven residues are conserved in all caspases, but only three of them (Gly, Gln, and Ser) are found in I-FLICE-1. Given this lack of conservation of key residues involved in catalysis and substrate binding it can be concluded that I-FLICE-1 is not a cysteine protease and is incapable of binding Asp at the P1 position. Interestingly, the DED domain of I-FLICE-1 was more related to the corresponding domains present in the viral DED-containing inhibitors K13, MC159, and E8, sharing 34%, 31%, and 33% identity (56%, 51%, and 44% similarity), respectively (Hu, S. *et al., J. Biol. Chem. 272*:9621-9624 (1997) and Thorne, M. *et al., Nature 386*:517-521 (1997)).

Co-immunoprecipitation analysis revealed the ability of I-FLICE-1 to bind FLICE and Mch4/FLICE2 but not FADD. In this respect, I-FLICE-1 resembles the viral DED-containing molecule E8 in that it binds FLICE but not FADD (Hu *et al., J. Biol. Chem. 272*:9621-9624 (1997); Bertin *et al., Proc. Natl. Acad. Sci. 94*:1172-1176 (1997)). Since there was no association between I-FLICE-1 and FADD, I-FLICE-1 was not recruited to the CD-95 or TNFR-1 signaling complex as evidenced by its inability to co-precipitate with these receptors.

Example 6: Cell Death Assay

Given the ability of the catalytically inactive I-FLICE-1 to complex with FLICE-like caspases, the inventors reasoned that I-FLICE-1 may be acting as a dominant negative inhibitor since the active form of all caspases is a tetramer derived from the processing of two zymogen forms to a four-chain assembly. It follows that a catalytically inert zymogen, such as I-FLICE-1, would be processed to inactive subunits that would result in the generation of a nonfunctional tetrameric protease. This mechanism predicts that I-FLICE-1 should inhibit

TNFR-1 and CD-95-induced apoptosis where FLICE-like caspases play an initiating role. The following cell death assay was performed.

Materials and Methods

Cell Death Assay - Human embryonic kidney 293 (for TNFR-1 killing) or 293 EBNA cells (for CD-95 killing) were transiently transfected with 0.1 μg of the reporter plasmid pCMV β-galactosidase plus 0.5μg of test plasmid in the presence or absence of 2.0μg of inhibitory plasmids. 22-24 hours after transfection, cells were fixed in 0.5% glutaraldehyde and stained with X-gal. Percentage of apoptotic cells was determined by calculating the fraction of membrane blebbed blue cells as a function of total blue cells. All assays were evaluated in duplicate and the mean and the standard deviation calculated.

Results

Consistent with the proposed mechanism, overexpression of I-FLICE-1 resulted in substantial inhibition of TNFR-1 induced cell death comparable to previously characterized inhibitors including CrmA, MC159, dominant negative FLICE (DNFLICE) and Mch4/FLICE2 (DNFLICE2) (see FIG. 6A). However, under the present experimental conditions, I-FLICE-1 appeared to be a less potent inhibitor of CD-95 induced cell death, possibly reflecting the more potent death signal that emanates from this receptor (see FIG. 6B).

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

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KENNY, JOSEPH J.

(ii) TITLE OF INVENTION: I-FLICE, A NOVEL INHIBITOR OF TUMOR NECROSIS FACTOR RECEPTOR-1 AND CD-95 INDUCED APOPTOSIS

(iii) NUMBER OF SEQUENCES: 32

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE: HEREWITH
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/034,205
(B) FILING DATE: 21-JAN-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/054,800
(B) FILING DATE: 05-AUG-1997

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2034 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 268..1707

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGACAGGACG AACTCCCCCA CTGGAAAGGA TTCTGAAAGA AATGAAGTCA GCCCTCAGAA     180

ATGAAGTTGA CTGCCTGCTG GCTTTCTGTT GACTGGCCCG GAGCTGTACT GCAAGACCCT     240

TGTGAGCTTC CCTAGTCTAA GAGTAGG ATG TCT GCT GAA GTC ATC CAT CAG        291
                              Met Ser Ala Glu Val Ile His Gln
                                1               5

GTT GAA GAA GCA CTT GAT ACA GAT GAG AAG GAG ATG CTG CTC TTT TTG      339
Val Glu Glu Ala Leu Asp Thr Asp Glu Lys Glu Met Leu Leu Phe Leu
     10                  15                  20

TGC CGG GAT GTT GCT ATA GAT GTG GTT CCA CCT AAT GTC AGG GAC CTT      387
Cys Arg Asp Val Ala Ile Asp Val Val Pro Pro Asn Val Arg Asp Leu
 25                  30                  35                  40

CTG GAT ATT TTA CGG GAA AGA GGT AAG CTG TCT GTC GGG GAC TTG GCT      435
Leu Asp Ile Leu Arg Glu Arg Gly Lys Leu Ser Val Gly Asp Leu Ala
                 45                  50                  55

GAA CTG CTC TAC AGA GTG AGG CGA TTT GAC CTG CTC AAA CGT ATC TTG      483
Glu Leu Leu Tyr Arg Val Arg Arg Phe Asp Leu Leu Lys Arg Ile Leu
             60                  65                  70

AAG ATG GAC AGA AAA GCT GTG GAG ACC CAC CTG CTC AGG AAC CCT CAC      531
Lys Met Asp Arg Lys Ala Val Glu Thr His Leu Leu Arg Asn Pro His
         75                  80                  85

CTT GTT TCG GAC TAT AGA GTG CTG ATG GCA GAG ATT GGT GAG GAT TTG      579
Leu Val Ser Asp Tyr Arg Val Leu Met Ala Glu Ile Gly Glu Asp Leu
     90                  95                 100

GAT AAA TCT GAT GTG TCC TCA TTA ATT TTC CTC ATG AAG GAT TAC ATG      627
Asp Lys Ser Asp Val Ser Ser Leu Ile Phe Leu Met Lys Asp Tyr Met
105                 110                 115                 120

GGC CGA GGC AAG ATA AGC AAG GAG AAG AGT TTC TTG GAC CTT GTG GTT      675
Gly Arg Gly Lys Ile Ser Lys Glu Lys Ser Phe Leu Asp Leu Val Val
                125                 130                 135
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GAG TTG GAG AAA CTA AAT CTG GTT GCC CCA GAT CAA CTG GAT TTA TTA          723
Glu Leu Glu Lys Leu Asn Leu Val Ala Pro Asp Gln Leu Asp Leu Leu
            140                 145                 150

GAA AAA TGC CTA AAG AAC ATC CAC AGA ATA GAC CTG AAG ACA AAA ATC          771
Glu Lys Cys Leu Lys Asn Ile His Arg Ile Asp Leu Lys Thr Lys Ile
        155                 160                 165

CAG AAG TAC AAG CAG TCT GTT CAA GGA GCA GGG ACA AGT TAC AGG AAT          819
Gln Lys Tyr Lys Gln Ser Val Gln Gly Ala Gly Thr Ser Tyr Arg Asn
    170                 175                 180

GTT CTC CAA GCA GCA ATC CAA AAG AGT CTC AAG GAT CCT TCA AAT AAC          867
Val Leu Gln Ala Ala Ile Gln Lys Ser Leu Lys Asp Pro Ser Asn Asn
185                 190                 195                 200

TTC AGG CTC CAT AAT GGG AGA AGT AAA GAA CAA AGA CTT AAG GAA CAG          915
Phe Arg Leu His Asn Gly Arg Ser Lys Glu Gln Arg Leu Lys Glu Gln
                205                 210                 215

CTT GGC GCT CAA CAA GAA CCA GTG AAG AAA TCC ATT CAG GAA TCA GAA          963
Leu Gly Ala Gln Gln Glu Pro Val Lys Lys Ser Ile Gln Glu Ser Glu
            220                 225                 230

GCT TTT TTG CCT CAG AGC ATA CCT GAA GAG AGA TAC AAG ATG AAG AGC         1011
Ala Phe Leu Pro Gln Ser Ile Pro Glu Glu Arg Tyr Lys Met Lys Ser
        235                 240                 245

AAG CCC CTA GGA ATC TGC CTG ATA ATC GAT TGC ATT GGC AAT GAG ACA         1059
Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp Cys Ile Gly Asn Glu Thr
    250                 255                 260

GAG CTT CTT CGA GAC ACC TTC ACT TCC CTG GGC TAT GAA GTC CAG AAA         1107
Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu Gly Tyr Glu Val Gln Lys
265                 270                 275                 280

TTC TTG CAT CTC AGT ATG CAT GGT ATA TCC CAG ATT CTT GGC CAA TTT         1155
Phe Leu His Leu Ser Met His Gly Ile Ser Gln Ile Leu Gly Gln Phe
                285                 290                 295

GCC TGT ATG CCC GAG CAC CGA GAC TAC GAC AGC TTT GTG TGT GTC CTG         1203
Ala Cys Met Pro Glu His Arg Asp Tyr Asp Ser Phe Val Cys Val Leu
            300                 305                 310

GTG AGC CGA GGA GGC TCC CAG AGT GTG TAT GGT GTG GAT CAG ACT CAC         1251
Val Ser Arg Gly Gly Ser Gln Ser Val Tyr Gly Val Asp Gln Thr His
        315                 320                 325

TCA GGG CTC CCC CTG CAT CAC ATC AGG AGG ATG TTC ATG GGA GAT TCA         1299
Ser Gly Leu Pro Leu His His Ile Arg Arg Met Phe Met Gly Asp Ser
    330                 335                 340

TGC CCT TAT CTA GCA GGG AAG CCA AAG ATG TTT TTT ATT CAG AAC TAT         1347
Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met Phe Phe Ile Gln Asn Tyr
345                 350                 355                 360

GTG GTG TCA GAG GGC CAG CTG GAG GAC AGC AGC CTC TTG GAG GTG GAT         1395
Val Val Ser Glu Gly Gln Leu Glu Asp Ser Ser Leu Leu Glu Val Asp
                365                 370                 375

GGG CCA GCG ATG AAG AAT GTG GAA TTC AAG GCT CAG AAG CGA GGG CTG         1443
Gly Pro Ala Met Lys Asn Val Glu Phe Lys Ala Gln Lys Arg Gly Leu
            380                 385                 390
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TGC ACA GTT CAC CGA GAA GCT GAC TTC TTC TGG AGC CTG TGT ACT GCG      1491
Cys Thr Val His Arg Glu Ala Asp Phe Phe Trp Ser Leu Cys Thr Ala
        395                 400                 405

GAC ATG TCC CTG CTG GAG CAG TCT CAC AGC TCA CCG TCC CTG TAC CTG      1539
Asp Met Ser Leu Leu Glu Gln Ser His Ser Ser Pro Ser Leu Tyr Leu
    410                 415                 420

CAG TGC CTC TCC CAG AAA CTG AGA CAA GAA AGA AAA CGC CCA CTC CTG      1587
Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu Arg Lys Arg Pro Leu Leu
425                 430                 435                 440

GAT CTT CAC ATT GAA CTC AAT GGC TAC ATG TAT GAT TGG AAC AGC AGA      1635
Asp Leu His Ile Glu Leu Asn Gly Tyr Met Tyr Asp Trp Asn Ser Arg
                445                 450                 455

GTT TCT GCC AAG GAG AAA TAT TAT GTC TGG CTG CAG CAC ACT CTG AGA      1683
Val Ser Ala Lys Glu Lys Tyr Tyr Val Trp Leu Gln His Thr Leu Arg
            460                 465                 470

AAG AAA CTT ATC CTC TCC TAC ACA TAAGAAACCA AAAGGCTGGG CGTAGTGGCT     1737
Lys Lys Leu Ile Leu Ser Tyr Thr
        475                 480

CGCACCTGTA ATCCCAGCAC TTTGGGAGGC CAAGGAGGGC GGATCACTTC AGGTCAGGAG    1797

TTCGAGACCA GCCTGGCCAA CATGGTAAAC GCTGTCCCTA GTAAGAGTGC AAAAATTAGC    1857

TGGGTGTGGG TGTGGGTACC TGTGTTCCCA GTTACTTGGG AGGCTGAGGT GGGAGGATCT    1917

TTTGAACCCA GGAGTTCAGG GTCATAGCAT GCTGTGATTG TGCCTACGAA TAGCCACTGC    1977

ATACCAACCT GGGCAATATA GCAAGATCCC ATCTTTTTAA AAAAAAAAAA AAAAAAA       2034
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(2) INFORMATION FOR SEQ ID NO:2:

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      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 480 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
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Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
            20                  25                  30

Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
        35                  40                  45

Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
    50                  55                  60

Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu
65                  70                  75                  80

Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu
                85                  90                  95
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Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
            100                 105                 110

Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
        115                 120                 125

Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
    130                 135                 140

Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
145                 150                 155                 160

Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
                165                 170                 175

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            180                 185                 190

Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser
        195                 200                 205

Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val
    210                 215                 220

Lys Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro
225                 230                 235                 240

Glu Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile
                245                 250                 255

Ile Asp Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr
            260                 265                 270

Ser Leu Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly
        275                 280                 285

Ile Ser Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp
    290                 295                 300

Tyr Asp Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser
305                 310                 315                 320

Val Tyr Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile
                325                 330                 335

Arg Arg Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro
            340                 345                 350

Lys Met Phe Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu
        355                 360                 365

Asp Ser Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu
    370                 375                 380

Phe Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp
385                 390                 395                 400

Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser
                405                 410                 415

His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg
            420                 425                 430
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Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly
435 440 445

Tyr Met Tyr Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr
450 455 460

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465 470 475 480

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 478 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asp Phe Ser Arg Asn Leu Tyr Asp Ile Gly Glu Gln Leu Asp Ser
1 5 10 15

Glu Asp Leu Ala Ser Leu Lys Phe Leu Ser Leu Asp Tyr Ile Pro Gln
20 25 30

Arg Lys Gln Glu Pro Ile Lys Asp Ala Leu Met Leu Phe Gln Arg Leu
35 40 45

Gln Glu Lys Arg Met Leu Glu Glu Ser Asn Leu Ser Phe Leu Lys Glu
50 55 60

Leu Leu Phe Arg Ile Asn Arg Leu Asp Leu Leu Ile Thr Tyr Leu Asn
65 70 75 80

Thr Arg Lys Glu Glu Met Glu Arg Glu Leu Gln Thr Pro Gly Arg Ala
85 90 95

Gln Ile Ser Ala Tyr Arg Val Met Leu Tyr Gln Ile Ser Glu Glu Val
100 105 110

Ser Arg Ser Glu Leu Arg Ser Phe Lys Phe Leu Leu Gln Glu Glu Ile
115 120 125

Ser Lys Cys Lys Leu Asp Asp Asp Met Asn Leu Leu Asp Ile Phe Ile
130 135 140

Glu Met Glu Lys Arg Val Ile Leu Gly Glu Gly Lys Leu Asp Ile Leu
145 150 155 160

Lys Arg Val Cys Ala Gln Ile Asn Lys Ser Leu Leu Lys Ile Ile Asn
165 170 175

Asp Tyr Glu Glu Phe Ser Lys Glu Arg Ser Ser Ser Leu Glu Gly Ser
180 185 190

Pro Asp Glu Phe Ser Asn Gly Glu Glu Leu Cys Gly Val Met Thr Ile
195 200 205

Ser Asp Ser Pro Arg Glu Gln Asp Ser Glu Ser Gln Thr Leu Asp Lys
    210   215   220

Val Tyr Gln Met Lys Ser Lys Pro Arg Gly Tyr Cys Leu Ile Ile Asn
225   230   235   240

Asn His Asn Phe Ala Lys Ala Arg Glu Lys Val Pro Lys Leu His Ser
                245   250   255

Ile Arg Asp Arg Asn Gly Thr His Leu Asp Ala Gly Leu Thr Thr Thr
            260   265   270

Phe Glu Glu Leu His Phe Glu Ile Lys Pro His Asp Asp Cys Thr Val
        275   280   285

Glu Gln Ile Tyr Glu Ile Leu Lys Ile Tyr Gln Leu Met Asp His Ser
    290   295   300

Asn Met Asp Cys Phe Ile Cys Cys Ile Leu Ser His Gly Asp Lys Gly
305   310   315   320

Ile Ile Tyr Gly Thr Asp Gly Gln Glu Pro Pro Ile Tyr Glu Leu Thr
                325   330   335

Ser Gln Phe Thr Gly Leu Lys Cys Pro Ser Leu Ala Gly Lys Pro Lys
            340   345   350

Val Phe Phe Ile Gln Ala Cys Gln Gly Asp Asn Tyr Gln Lys Gly Ile
        355   360   365

Pro Val Glu Thr Asp Ser Glu Glu Gln Pro Tyr Leu Glu Met Asp Leu
    370   375   380

Ser Ser Pro Gln Thr Arg Tyr Ile Pro Asp Glu Ala Asp Phe Leu Leu
385   390   395   400

Gly Met Ala Thr Val Asn Asn Cys Val Ser Tyr Arg Asn Pro Ala Glu
                405   410   415

Gly Thr Trp Tyr Ile Gln Ser Leu Cys Gln Ser Leu Arg Glu Arg Cys
            420   425   430

Pro Arg Gly Asp Asp Ile Leu Thr Ile Leu Thr Glu Val Asn Tyr Glu
        435   440   445

Val Ser Asn Lys Asp Asp Lys Lys Asn Met Gly Lys Gln Met Pro Gln
    450   455   460

Pro Thr Phe Thr Leu Arg Lys Lys Leu Val Phe Pro Ser Asp
465   470   475

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 479 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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1               5                   10                  15

Lys Val Ser Phe Arg Glu Lys Leu Leu Ile Ile Asp Ser Asn Leu Gly
            20                  25                  30

Val Gln Asp Val Glu Asn Leu Lys Phe Leu Cys Ile Gly Leu Val Pro
        35                  40                  45

Asn Lys Lys Leu Glu Lys Ser Ser Ser Ala Ser Asp Val Phe Glu His
    50                  55                  60

Leu Leu Ala Glu Asp Leu Leu Ser Glu Glu Asp Pro Phe Phe Leu Ala
65                  70                  75                  80

Glu Leu Leu Tyr Ile Ile Arg Gln Lys Lys Leu Leu Gln His Leu Asn
                85                  90                  95

Cys Thr Lys Glu Glu Val Glu Arg Leu Leu Pro Thr Arg Gln Arg Val
            100                 105                 110

Ser Leu Phe Arg Asn Leu Leu Tyr Glu Leu Ser Glu Gly Ile Asp Ser
        115                 120                 125

Glu Asn Leu Lys Asp Met Ile Phe Leu Leu Lys Asp Ser Leu Pro Lys
    130                 135                 140

Thr Glu Met Thr Ser Leu Ser Phe Leu Ala Phe Leu Glu Lys Gln Gly
145                 150                 155                 160

Lys Ile Asp Glu Asp Asn Leu Thr Cys Leu Glu Asp Leu Cys Lys Thr
                165                 170                 175

Val Val Pro Lys Leu Leu Arg Asn Ile Glu Lys Tyr Lys Arg Glu Lys
            180                 185                 190

Ala Ile Gln Ile Val Thr Pro Pro Val Asp Lys Glu Ala Glu Ser Tyr
        195                 200                 205

Gln Gly Glu Glu Glu Leu Val Ser Gln Thr Asp Val Lys Thr Phe Leu
    210                 215                 220

Glu Ala Leu Pro Arg Ala Ala Val Tyr Arg Met Asn Arg Asn His Arg
225                 230                 235                 240

Gly Leu Cys Val Ile Val Asn Asn His Ser Phe Thr Ser Leu Lys Asp
                245                 250                 255

Arg Gln Gly Thr His Lys Asp Ala Glu Ile Leu Ser His Val Phe Gln
            260                 265                 270

Trp Leu Gly Phe Thr Val His Ile His Asn Asn Val Thr Lys Val Glu
        275                 280                 285

Met Glu Met Val Leu Gln Lys Gln Lys Cys Asn Pro Ala His Ala Asp
    290                 295                 300

Gly Asp Cys Phe Val Phe Cys Ile Leu Thr His Gly Arg Phe Gly Ala
305                 310                 315                 320

Val Tyr Ser Ser Asp Glu Ala Leu Ile Pro Ile Arg Glu Ile Met Ser
```

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                325                 330                 335

His Phe Thr Ala Leu Gln Cys Pro Arg Leu Ala Glu Lys Pro Lys Leu
            340                 345                 350

Phe Phe Ile Gln Ala Cys Gln Gly Glu Glu Ile Gln Pro Ser Val Ser
        355                 360                 365

Ile Glu Ala Asp Ala Leu Asn Pro Glu Gln Ala Pro Thr Ser Leu Gln
    370                 375                 380

Asp Ser Ile Pro Ala Glu Ala Asp Phe Leu Leu Gly Leu Ala Thr Val
385                 390                 395                 400

Pro Gly Tyr Val Ser Phe Arg His Val Glu Glu Gly Ser Trp Tyr Ile
                405                 410                 415

Gln Ser Leu Cys Asn His Leu Lys Lys Leu Val Pro Arg His Glu Asp
            420                 425                 430

Ile Leu Ser Ile Leu Thr Ala Val Asn Asp Asp Val Ser Arg Arg Val
        435                 440                 445

Asp Lys Gln Gly Thr Lys Lys Gln Met Pro Gln Pro Ala Phe Thr Leu
    450                 455                 460

Arg Lys Lys Leu Val Phe Pro Val Pro Leu Asp Ala Leu Ser Ile
465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2597 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
- (A) NAME/KEY: CDS
- (B) LOCATION: 304..1347

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GCGAGCTTGC AGCCTCACCG ACGAGTCTCA ACTAAAAGGG ACTCCCGGAG CTAGGGGTGG      60

GGACTCGGCC TCACACAGTG ATTGCCGGCT ATTGGACTTT TGTCCAGTGA CAGCTGAGAC     120

AACAAGGACC ACGGGAGGAG GTGTAGGAGA GAAGCGCCGC GAACAGGCAT CGCCCAGCAC     180

CAAGTCCGCT TCCAGGCTTT CGGTTTCTTT GCCTCCATCT TGGGTGCGCC TTCCCGGCGT     240

CTAGGGGAGC GAAGGCTGAG GTGGCAGCGG CAGGAGAGTC CGGCCGCGAC AGGACGAGTG     300

CTG ATG GCA GAG ATT GGT GAG GAT TTG GAT AAA TCT GAT GTG TCC TCA       348
    Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser
                    5                  10                  15

TTA ATT TTC CTC ATG AAG GAT TAC ATG GGC CGA GGC AAG ATA AGC AAG       396
Leu Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys
```

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                    20                  25                  30

GAG AAG AGT TTC TTG GAC CTT GTG GTT GAG TTG GAG AAA CTA AAT CTG      444
Glu Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu
             35                  40                  45

GTT GCC CCA GAT CAA CTG GAT TTA TTA GAA AAA TGC CTA AAG AAC ATC      492
Val Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile
         50                  55                  60

CAC AGA ATA GAC CTG AAG ACA AAA ATC CAG AAG TAC AAG CAG TCT GTT      540
His Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val
     65                  70                  75

CAA GGA GCA GGG ACA AGT TAC AGG AAT GTT CTC CAA GCA GCA ATC CAA      588
Gln Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln
 80                  85                  90                  95

AAG AGT CTC AAG GAT CCT TCA AAT AAC TTC AGG GAA GAA CCA GTG AAG      636
Lys Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Glu Glu Pro Val Lys
                100                 105                 110

AAA TCC ATT CAG GAA TCA GAA GCT TTT TTG CCT CAG AGC ATA CCT GAA      684
Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro Glu
            115                 120                 125

GAG AGA TAC AAG ATG AAG AGC AAG CCC CTA GGA ATC TGC CTG ATA ATC      732
Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile
        130                 135                 140

GAT TGC ATT GGC AAT GAG ACA GAG CTT CTT CGA GAC ACC TTC ACT TCC      780
Asp Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser
    145                 150                 155

CTG GGC TAT GAA GTC CAG AAA TTC TTG CAT CTC AGT ATG CAT GGT ATA      828
Leu Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile
160                 165                 170                 175

TCC CAG ATT CTT GGC CAA TTT GCC TGT ATG CCC GAG CAC CGA GAC TAC      876
Ser Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr
                180                 185                 190

GAC AGC TTT GTG TGT GTC CTG GTG AGC CGA GGA GGC TCC CAG AGT GTG      924
Asp Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val
            195                 200                 205

TAT GGT GTG GAT CAG ACT CAC TCA GGG CTC CCC CTG CAT CAC ATC AGG      972
Tyr Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg
        210                 215                 220

AGG ATG TTC ATG GGA GAT TCA TGC CCT TAT CTA GCA GGG AAG CCA AAG     1020
Arg Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys
    225                 230                 235

ATG TTT TTT ATT CAG AAC TAT GTG GTG TCA GAC GGC CAG CTG GAG GAC     1068
Met Phe Phe Ile Gln Asn Tyr Val Val Ser Asp Gly Gln Leu Glu Asp
240                 245                 250                 255

AGC AGC CTC TTG GAG GTG GAT GGG CCA GCG ATG AAG AAT GTG GAA TTC     1116
Ser Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe
                260                 265                 270

AAG GCT CAG AAG CGA GGG CTG TGC ACA GTT CAC CGA GAA GCT GAC TTC     1164
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Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe
            275                 280                 285

TTC TGG AGC CTG TGT ACT GCG GAC ATG TCC CTG CTG GAG CAG TCT CAC      1212
Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His
        290                 295                 300

AGC TCA CCG TCC CTG TAC CTG CAG TGC CTC TCC CAG AAA CTG AGA CAA      1260
Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln
    305                 310                 315

GAA AGG GGG ACA ATT CCC GGA AGT GGA ATT ACA GAG TCA AAG GAC ATG      1308
Glu Arg Gly Thr Ile Pro Gly Ser Gly Ile Thr Glu Ser Lys Asp Met
320                 325                 330                 335

CAT TTT TCA AGC CTC GGA TGC ATC TTA CTA GAT GTC CTA TAGGATGGTC       1357
His Phe Ser Ser Leu Gly Cys Ile Leu Leu Asp Val Leu
                340                 345

ATATCAGCTT TATAGGAGAG TAGCTGTGTC CCTGAATTCT CCCTGACACT GCATGCTCTT    1417

ATATTTCCTC AAGTTTTGAC AATTTGATAG GTGAAAAGTG GTATCTGACT GTTCAGATCT    1477

GGAAGGCTTT GTTATATAAA CATTTTTTTA ATGTTTATTG GCAAGAATAC TTTTCTAAGA    1537

GAAACATCAG TGAGCTGGTT TCCATTTAAG CTGAATGAAG CCACAATGTA CCTCAAGTAT    1597

AAGATTAACT GGCCTTTTTC AGTTGCACTC TAATTACAAT TTAGAATGAT GTTTCTGAGC    1657

CACCTGTCAA ATGCATTCTG GGCTGTACCT CTGCGTACCC CAGGAATAAA TCTCATGGCC    1717

TTCTTTACCT GGCCTCCTTA GTGGTGGCCC AGCAGGAAGC GGGGGTTAGA GCAGGAGCCA    1777

CTCAGCCTTC CAAGATAGAT ACTCCATGGG CCGGTGGTAT TACTGGCCTT TTGAGCCCAT    1837

CCCCATTTGC ATAGATGATC CACGTGGGTT ATCATCTGGC TGGTATGTTC CCAGAGTGAA    1897

ACTCAGCAGC CCCTTGAGGG AGGGGATGGT GGCCATCAGG CCAGAGTATT GCAAGTTAGT    1957

TTGGATCATT TGCTAAGCAG CTTGTGGTGC CTTCAGAAAG GAACAGTTTC AAAGAACTTT    2017

CACATCTGTT GGCTCATTTC GCCCTAATGA CAGTCTTCTC TTTGATATTT GCATGGCATT    2077

AAATTTTGCC TTTCTTGTTT TCTCCAGAAA ACGCCCACTC CTGGATCTTC ACATTGAACT    2137

CAATGGCTAC ATGTATGATT GGAACAGCAG AGTTTCTGCC AAGGAGAAAT ATTATGTCTG    2197

GCTGCAGCAC ACTCTGAGAA AGAAACTTAT CTCTCCTACA CATAAGAAAC CAAAAGGCTG    2257

GGCGTAGTGG CTCGCACCTG TGATCCCAGC ACTTTGGGAG GCCGAGGAGG GCGGATCACT    2317

TCAGGTCGGG AGTTCGAGAC CAGCCTGGCC AGCATGTGAA CGCTGTCCCT AGTAGAAGTG    2377

CAAAAATTGG CTGGTGTGGG TGTGGGTACC CTGTATTCCC AGTTGCTTGG GGGGCTGAGG    2437

TGGGAGGATC TTTTGACCCC AGGAGTTCAG GGTCATAGCA TGCTGTGATT GTGCCTACGA    2497

ATAGCCACTG CATACCAACC TGGGCAATAT AGCAAGATCC CATCTCTTTA AAAAAAAAAA    2557

AAAAAGGACA GGAACTATCT TAAAAAAAAA AAAAAAAAAA                          2597
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
 1               5                  10                  15

Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
            20                  25                  30

Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
        35                  40                  45

Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
    50                  55                  60

Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
65                  70                  75                  80

Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys
                85                  90                  95

Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Glu Glu Pro Val Lys Lys
            100                 105                 110

Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro Glu Glu
        115                 120                 125

Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp
    130                 135                 140

Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu
145                 150                 155                 160

Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile Ser
                165                 170                 175

Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr Asp
            180                 185                 190

Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val Tyr
        195                 200                 205

Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg Arg
    210                 215                 220

Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met
225                 230                 235                 240

Phe Phe Ile Gln Asn Tyr Val Val Ser Asp Gly Gln Leu Glu Asp Ser
                245                 250                 255

Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe Lys
            260                 265                 270

Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe Phe
        275                 280                 285
```

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Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His Ser
    290                 295                 300

Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu
305                 310                 315                 320

Arg Gly Thr Ile Pro Gly Ser Gly Ile Thr Glu Ser Lys Asp Met His
                325                 330                 335

Phe Ser Ser Leu Gly Cys Ile Leu Leu Asp Val Leu
            340                 345
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCCCATGGC TGAAGTCATC CATCAG 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCAAGCTTG TGCTGGGATT ACAGGTG 27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCCCATGGA GATTGGTGAG GATTTG 26

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCAAGCTTA GAGCATGCAG TGTCAG 26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGGATCCG CCATCATGTC TGCTGAAGTC ATC 33

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGGTACCG TGCTGGGATT ACAGGTG 27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCGGATCCG CCATCATGGC AGAGATTGGT GAG 33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGCGGTACCA GAGCATGCAG TGTCAG 26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCCCCGGGG CCATCATGTC TGCTGAAGTC ATC 33

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAG TGCTGGGATT ACAGGTG 57

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGATCCG CCATCATGTC TGCTGAAGTC ATC 33

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCGGTACCG TGCTGGGATT ACAGGTG 27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGCGGATCCG CCATCATGGC AGAGATTGGT GAG 33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAA GAGCATGCAG TGTCAG 56

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCGGATCCG CCATCATGGC AGAGATTGGT GAG 33

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGCGGTACCA GAGCATGCAG TGTCAG 26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 414 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATTCGGCAC GAGGGNGGAC TTGGCTGAAC TGCTCTACAG AGTGAGGCGA TTTGACCTGC 60

TCAAACGTAT CTTGAAGATG GACAGAAAAG CTGTGGAGAC CCACCTGCTC AGGAACCCTC 120

ACCTTGTTTC GGACTATAGA GTGCTGATGG CAGAGATTGG TGAGGATTTG GATAAATCTG 180

ATGTGTCCTC ATTAATTTTC CTCATGAAGG ATTACATGGG CCGAGGCAAG ATAAGCAAGG 240

AGAAGAGTTT CTTGGGACCT TGGTGGTTGA GTTGGGAGAA ACTAAATCTG GTTTGCCCCA 300

GATCAACTNG GGATTTNTTA GGAAAAATGC CTAAAGAACA TNCACAGGAT AGACCTGNAG 360

ACAAAANTCC AGNAGTACAN GCAGTNTGTT CAGGGAGCAG GGACAATTNC AGGA 414

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 393 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
TGCCAAGGAG AAATATTATG TCTGGCTGCA GCACACTCTG NGAAAGAAAC TTATCCTCTC    60

CTACACATAA GAAACCNAAA GGCTGGGCGT AGTGGCTCAC GCCTGTNAAT CCCAGCACTT   120

TGGGAGGCCA AGGAGGGCAG ATCACTTCAG GTCAGGAGTT CGAGACCAGC CTGGCCAACA   180

TGGTAAACGC TGTCCCTAGT AAAANTACAA AANTTAGCTG GGTGTGGGTG TGGGTACCTG   240

TGTTCCCAGT TACTTGGGAG GCTGAGGTGG GAGGATCTTT TGGAACCCAG GAGTTTCAGG   300

GTCATAGCAT GCTGTGNTTG TGCCCTNACG AATTAGCCAC TGCATTACCA ACCTGGGGCA   360

ATNTTAGGCA AGATCCCATN TNTTTTAAAA AAA                                393
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 309 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
TGGATCTTCA CATTGAACTC AATGGCTACA TGTATGATTG GAACAGCAGA GTTTCTGCCA    60

AGGAGAAATA TTATGTCTGG CTGCAGCACA CTCTGAGAAA GAAACTTATC CTCTCCTACA   120

CATAAGAAAC CAAAAGGCTG GGCGTAGTGG CTCACGCCTG TGATCCCAGC ACTTTGGGAG   180

GCCGGGGAGG GCAGATCACT TCAGGTCAGG AGTTCGAGAC CGGCCTGGNC AACATGGTAG   240

ACGCTGTCCC TAGTAAAAAT GCAAAAGTTG GCTGGGTGTG GGTGTNGGTA CCTGTGTTCC   300

CAGTTGCTT                                                           309
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 500 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AATTCGGCAG AGCTCACTCA GGGCTCCCCC TGCATCACAT CAGGAGGATG TTCATGGGAG 60

ATTCATGCCC TTATCTAGCA GGGAAGCCAA AGATGTTTTT TATTCAGAAC TATGTGNTGT 120

CAGAGGGCCA GCTGGAGGAC AGCAGCCTCT TGGAGGTGGA TGGGCCAGCN ATGAAGAATG 180

TGGAATTCAA GGCTCAGAAG CGAGGGCTGT GCACAGTTCA CCGAGNAAGC TGACTTCTTC 240

TGGAGCCTGT GTAATGCGGA CATGTCCCTG CTTGGAGCAA TCTTCANAGG TTCANCGTCC 300

CTGTNACCTG CATGCCTTTT CCCAGAAACT GNGACAAGNA AGAAAACGNC CANTNCTGGG 360

GNTNTTTCAC ATTGGAACTC AATGGTTACA ANTTATGNTT GGGGNCAACA ANTTTTTTGC 420

CAAGGGGGAA TTTTTTGTTT TGGGNTGNAG AAAAATTTNG GGAAAGAANT TTTTCCCTTN 480

CNNNAAATTA GGNACCCAAA 500

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATTCTGAAAA AGAATGTGGG GTTTCCTTGC AGATGAGTTC ATCTGTTGTT TCATTTCCTT 60

TACAATAACT CCCCCACTGG AAAGGATTCT GAAAGAAATG AAGTCAGCCC TCAGAAATGA 120

AGTTGACTGC CTGCTGGCTT TCTGTTGACT GGCCTGGAGC TGTACTGCAA GACCCTTGTG 180

AGCTTCCCTA GTCTAAGAGT AGGATGTCTG CTGAAGTCAT CCATCAGGTT GAAGAAGCAC 240

TTGATACAGA TGAGAAGGAG ATGCTGCTCT TTTTGTGCCG GGATGTTGCT ATAGATGTGG 300

TTCCACCTAA TGTCAGGGAC CTTC 324

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 389 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

NAATTCGGCA GAGANAAGAG TCTCAAGGAT CCTTCAAATA ACTTCAGGCT CCATAATGGG 60

AGAAGTAAAG AACAAAGACT TAAGGAACAG CTTGGCGCTC AACAAGAACC AGTGNAAGAA 120

ATCCATTCAG GAATCAGAAG CTTTTTTGCC TCAGAGCATA CCTGAAGAGA GATACAAGAT 180

GAAGAGCAAG CCCCTAGGGA ATCTGCCTGA TAAATCGATT GCATTGGCAA TGAGGACAGA 240

GCTTCTTCGG GGACACCTTC ACTTCCCTGG GCTTATGAAG TNCCAGGAAA TTCTTGCATC 300

TCAGTATGCA TGGTATTNTC CCAGATTTTT TGGGNCCAAT TTGCCCGTTA TGNCCNGGGC 360

ANCNGGGGAT TTANGACAAT TTTGTGGTG 389

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 308 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATTCTGAAAA AGANTGNGGG GTTTCCTTGC AGATGAGTTC ATCTNTTGTT TCATTTCCTT 60

TACAATAACT CCCCCACTGG AAAGGATTCT GAAAGNAATG AAGTCAGCCC TCAGAAATGA 120

AGTTGNCTGC CTGCTGGCTT TCTGTTGACT GGCCTGGAGC TGTACTGCAA GACCCTTGTG 180

AGCTTCCCTA GTCTAAGAGT AGGATGTCTG CTGAAGTCAT CCATCAGGTT GAAGAAGCAC 240

TTGATACAGA TGAGAAGGAG ATGCTGCTCT TTTTGTGCCG GGATGTTTGC TATAGATGTG 300

GTTCCACC 308

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 297 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATTCTGAAAA AGAATGTGGG GTTTCCTNGC AGATGAGTTC ATCTGTTGTT TCATTTCCTT 60

TACAATAACT CCCCCACTGG AAAGGATTCT GAAAGAAATG AAGTCAGCCC TCAGAAATGA 120

AGTTGACTGC CTGCTGGCTT TCTGTTGACT GGCCTGGAGC TGTACTGCAA GACCCTTGTG 180

AGCTTCCCTA GTCTAAGAGT AGGATGTCTG CTGAAGTCAT CCATCAGGTT GAAGAAGCAC 240

TTGATACAGA TGAGAAGGAG ATGCTGCTCT TTTTTGTGCC GGGATGTTGC TATAGAT 297

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TATAGGATGG TCATATCAGC TTTATAGGAG AGTAGCTGTG TCCCTGAATT CTCCCTGACA 60

CTGCATGCTC TTATATTTCC TCAAGTTTTG ACAATTTGAT AGGTGAAAAG TGGTATCTGA 120

CTGTTCAGAT CTGGAAGGCT TTGTTATATA AACATTTTTT TAATGTTTAT TGGCAAGAAT 180

ACTTTTCTAA GAGAAACATC AGTGAGCTGG TTTCCATTTA AGCTGAATGA AGCCACAATG 240

TACCTCAAGT ATAAGGTTAA CTGGCCTTTT TTCAGTTGCA CTCTAATTAC AATTTAGAAT 300

GATGTTTCTG AGCCACCTGT CAAATGCATT CTGGGGCTGT ACCTCTTG 348

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TATAGGATGG TCATATCAGC TTTATAGGAG AGTAGCTGTG TCCCTGANTT CTCCCTGACA 60

CTGCATGCTC TTATATTTCC TCAAGTTTTG ACAATTTGAT AGGTGAAAAG TGGTATCTGA 120

CTGTNCAGAT CTGGAAGGCT TTGTTATATA AACATTTTTT TAATGTTTAT TGGCAAGAAT 180

ACTTTTCTAA GAGAAACATC AGTGAGCTGG TTTCCATTTA AGCTGAATGA AGCCACAATG 240

TACCTCANGT ATAAGGATTA ACTGGCCTTT TTCCAGTTGC ACTCTAATTA CAATTTTAGA 300

ATGATGTTCN GAGGCCACCT GTCAAATGCA TTC 333

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 28.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209038

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

DNA Plasmid 1631454

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

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Authorized officer Sonya Barnes PCT International Division	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 5.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209041

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

DNA Plasmid 1789630

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer PCT International Division	Authorized officer

What Is Claimed Is:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 480 in SEQ ID NO:2;

(b) a nucleotide sequence encoding a polypeptide comprising amino acids from about 2 to about 480 in SEQ ID NO:2;

(c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 348 in SEQ ID NO:6;

(d) a nucleotide sequence encoding a polypeptide comprising amino acids from about 2 to about 348 in SEQ ID NO:6;

(e) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209038;

(f) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209041; and

(g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), or (f).

2. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), or (g) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

3. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

4. A recombinant vector produced by the method of claim 3.

5. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 4 into a host cell.

6. A recombinant host cell produced by the method of claim 5.

7. A recombinant method for producing an I-FLICE-1 or I-FLICE-2 polypeptide, comprising culturing the recombinant host cell of claim 6 under conditions such that said polypeptide is expressed and recovering said polypeptide.

8. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 75 in SEQ ID NO:2;

(b) a nucleotide sequence encoding a polypeptide comprising amino acids from about 91 to about 171 in SEQ ID NO:2;

(c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 172 to about 375 in SEQ ID NO:2;

(d) a nucleotide sequence encoding a polypeptide comprising amino acids from about 376 to about 480 in SEQ ID NO:2;

(e) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 75 in SEQ ID NO:6;

(f) a nucleotide sequence encoding a polypeptide comprising amino acids from about 76 to about 252 in SEQ ID NO:6;

(f) a nucleotide sequence encoding a polypeptide comprising amino acids from about 253 to about 348 in SEQ ID NO:6; and

(g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), or (f).

9. An isolated I-FLICE-1 or I-FLICE-2 polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) amino acids from about 1 to about 480 in SEQ ID NO:2;

(b) amino acids from about 2 to about 480 in SEQ ID NO:2;

(c) amino acids from about 1 to about 348 in SEQ ID NO:6;

(d) amino acids from about 2 to about 348 in SEQ ID NO:6;

(e) the amino acid sequence of the I-FLICE-1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209038;

(f) the amino acid sequence of the I-FLICE-2 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209041; and

(g) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), or (f).

10. An isolated antibody that binds specifically to an I-FLICE-1 or I-FLICE-2 polypeptide of claim 9.

11. A method for treating diseases and disorders associated with apoptosis comprising administering to said individual a composition comprising an isolated polypeptide of claim 9.

12. A method useful during the diagnosis of diseases and disorders associated with aberrant cell survival in an individual comprising:

(a) measuring I-FLICE-1 or I-FLICE-2 gene expression level in cells or body fluid of said individual;

(b) comparing the I-FLICE-1 or I-FLICE-2 gene expression level of said individual with a standard I-FLICE-1 or I-FLICE-2 gene expression level, whereby an increase or decrease in the I-FLICE-1 or I-FLICE-2 gene expression level of said individual compared to said standard expression level is indicative of disease or disorder associated with aberrant cell survival.

13. An isolated nucleic acid molecule comprising a polynucleotide encoding an I-FLICE-1 or I-FLICE-2 polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 480 in SEQ ID NO:2;

(b) a nucleotide sequence encoding a polypeptide comprising amino acids from about 2 to about 480 in SEQ ID NO:2;

(c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 348 in SEQ ID NO:6;

(d) a nucleotide sequence encoding a polypeptide comprising amino acids from about 2 to about 348 in SEQ ID NO:6;

(e) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209038;

(f) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209041; and

(g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), or (f).

14. An isolated I-FLICE-1 or I-FLICE-2 polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:

(a) amino acids from about 1 to about 480 in SEQ ID NO:2;

(b) amino acids from about 2 to about 480 in SEQ ID NO:2;

(c) amino acids from about 1 to about 348 in SEQ ID NO:6;

(d) amino acids from about 2 to about 348 in SEQ ID NO:6;

(e) the amino acid sequence of the I-FLICE-1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209038;

(f) the amino acid sequence of the I-FLICE-2 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209041; and

(g) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), or (f).

15. An isolated I-FLICE-1 or I-FLICE-2 polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) amino acids from about 1 to about 75 in SEQ ID NO:2;

(b) amino acids from about 91 to about 171 in SEQ ID NO:2;

(c) amino acids from about 172 to about 375 in SEQ ID NO:2;

(d) amino acids from about 376 to about 480 in SEQ ID NO:2;

(e) amino acids from about 1 to about 75 in SEQ ID NO:6;

(f) amino acids from about 76 to about 252 in SEQ ID NO:6;

(g) amino acids from about 253 to about 348 in SEQ ID NO:6;

and

(h) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), or (g).

```
         10                            30                            50
CGATCGCCCAGCACCAAGTCCGCTTCCAGGCTTTCGGTTTCTTTGCCTCCATCTTGGGTG

         70                            90                           110
CGCCTTCCCGGCGTCTAGGGGAGCGAAGGCTGAGGTGGCAGCGGCAGGAGAGTCCGGCCG

        130                           150                           170
CGACAGGACGAACTCCCCCACTGGAAAGGATTCTGAAAGAAATGAAGTCAGCCCTCAGAA

        190                           210                           230
ATGAAGTTGACTGCCTGCTGGCTTTCTGTTGACTGGCCCGGAGCTGTACTGCAAGACCCT

        250                           270                           290
TGTGAGCTTCCCTAGTCTAAGAGTAGGATGTCTGCTGAAGTCATCCATCAGGTTGAAGAA
                            M  S  A  E  V  I  H  Q  V  E  E
        310                           330                           350
GCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTTTGTGCCGGGATGTTGCTATAGAT
A  L  D  T  D  E  K  E  M  L  L  F  L  C  R  D  V  A  I  D

        370                           390                           410
GTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAGAGGTAAGCTGTCT
V  V  P  P  N  V  R  D  L  L  D  I  L  R  E  R  G  K  L  S

        430                           450                           470
GTCGGGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGTATC
V  G  D  L  A  E  L  L  Y  R  V  R  R  F  D  L  L  K  R  I

        490                           510                           530
TTGAAGATGGACAGAAAAGCTGTGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCG
L  K  M  D  R  K  A  V  E  T  H  L  L  R  N  P  H  L  V  S

        550                           570                           590
GACTATAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCTCA
D  Y  R  V  L  M  A  E  I  G  E  D  L  D  K  S  D  V  S  S

        610                           630                           650
TTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTTTC
L  I  F  L  M  K  D  Y  M  G  R  G  K  I  S  K  E  K  S  F

        670                           690                           710
TTGGACCTTGTGGTTGAGTTGGAGAAACTAAATCTGGTTGCCCCAGATCAACTGGATTTA
L  D  L  V  V  E  L  E  K  L  N  L  V  A  P  D  Q  L  D  L

        730                           750                           770
TTAGAAAAATGCCTAAAGAACATCCACAGAATAGACCTGAAGACAAAAATCCAGAAGTAC
L  E  K  C  L  K  N  I  H  R  I  D  L  K  T  K  I  Q  K  Y

        790                           810                           830
AAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACAGGAATGTTCTCCAAGCAGCAATCCAA
K  Q  S  V  Q  G  A  G  T  S  Y  R  N  V  L  Q  A  A  I  Q

        850                           870                           890
AAGAGTCTCAAGGATCCTTCAAATAACTTCAGGCTCCATAATGGGAGAAGTAAAGAACAA
K  S  L  K  D  P  S  N  N  F  R  L  H  N  G  R  S  K  E  Q

        910                           930                           950
AGACTTAAGGAACAGCTTGGCGCTCAACAAGAACCAGTGAAGAAATCCATTCAGGAATCA
R  L  K  E  Q  L  G  A  Q  Q  E  P  V  K  K  S  I  Q  E  S

        970                           990                          1010
GAAGCTTTTTTGCCTCAGAGCATACCTGAAGAGAGATACAAGATGAAGAGCAAGCCCCTA
E  A  F  L  P  Q  S  I  P  E  E  R  Y  K  M  K  S  K  P  L
       1030                          1050                          1070
```

FIG.1A

```
GGAATCTGCCTGATAATCGATTGCATTGGCAATGAGACAGAGCTTCTTCGAGACACCTTC
G  I  C  L  I  I  D  C  I  G  N  E  T  E  L  L  R  D  T  F

       1090                1110                1130
ACTTCCCTGGGCTATGAAGTCCAGAAATTCTTGCATCTCAGTATGCATGGTATATCCCAG
T  S  L  G  Y  E  V  Q  K  F  L  H  L  S  M  H  G  I  S  Q

       1150                1170                1190
ATTCTTGGCCAATTTGCCTGTATGCCCGAGCACCGAGACTACGACAGCTTTGTGTGTGTC
I  L  G  Q  F  A  C  M  P  E  H  R  D  Y  D  S  F  V  C  V

       1210                1230                1250
CTGGTGAGCCGAGGAGGCTCCCAGAGTGTGTATGGTGTGGATCAGACTCACTCAGGGCTC
L  V  S  R  G  G  S  Q  S  V  Y  G  V  D  Q  T  H  S  G  L

       1270                1290                1310
CCCCTGCATCACATCAGGAGGATGTTCATGGGAGATTCATGCCCTTATCTAGCAGGGAAG
P  L  H  H  I  R  R  M  F  M  G  D  S  C  P  Y  L  A  G  K

       1330                1350                1370
CCAAAGATGTTTTTTATTCAGAACTATGTGGTGTCAGAGGGCCAGCTGGAGGACAGCAGC
P  K  M  F  F  I  Q  N  Y  V  V  S  E  G  Q  L  E  D  S  S

       1390                1410                1430
CTCTTGGAGGTGGATGGGCCAGCGATGAAGAATGTGGAATTCAAGGCTCAGAAGCGAGGG
L  L  E  V  D  G  P  A  M  K  N  V  E  F  K  A  Q  K  R  G

       1450                1470                1490
CTGTGCACAGTTCACCGAGAAGCTGACTTCTTCTGGAGCCTGTGTACTGCGGACATGTCC
L  C  T  V  H  R  E  A  D  F  F  W  S  L  C  T  A  D  M  S

       1510                1530                1550
CTGCTGGAGCAGTCTCACAGCTCACCGTCCCTGTACCTGCAGTGCCTCTCCCAGAAACTG
L  L  E  Q  S  H  S  S  P  S  L  Y  L  Q  C  L  S  Q  K  L

       1570                1590                1610
AGACAAGAAAGAAAACGCCCACTCCTGGATCTTCACATTGAACTCAATGGCTACATGTAT
R  Q  E  R  K  R  P  L  L  D  L  H  I  E  L  N  G  Y  M  Y

       1630                1650                1670
GATTGGAACAGCAGAGTTTCTGCCAAGGAGAAATATTATGTCTGGCTGCAGCACACTCTG
D  W  N  S  R  V  S  A  K  E  K  Y  Y  V  W  L  Q  H  T  L

       1690                1710                1730
AGAAAGAAACTTATCCTCTCCTACACATAAGAAACCAAAAGGCTGGGCGTAGTGGCTCGC
R  K  K  L  I  L  S  Y  T  *

       1750                1770                1790
ACCTGTAATCCCAGCACTTTGGGAGGCCAAGGAGGGCGGATCACTTCAGGTCAGGAGTTC

       1810                1830                1850
GAGACCAGCCTGGCCAACATGGTAAACGCTGTCCCTAGTAAGAGTGCAAAAATTAGCTGG

       1870                1890                1910
GTGTGGGTGTGGGTACCTGTGTTCCCAGTTACTTGGGAGGCTGAGGTGGGAGGATCTTTT

       1930                1950                1970
GAACCCAGGAGTTCAGGGTCATAGCATGCTGTGATTGTGCCTACGAATAGCCACTGCATA

       1990                2010                2030
CCAACCTGGGCAATATAGCAAGATCCCATCTTTTTAAAAAAAAAAAAAAAAAAA
```

FIG.1B

```
1    MD-----------------FSRNLYDIGEQLDSEDLASLK  huFLICE(U58143)
1    MKSQGQHWYSSSDKNCKVSFREKLLIIDSNLGVQDVENLK  HuMch4(U60519)
1    MSAEVIH------------------QVEEALDTDEKEMLL  HSALZ11Xprotein
1    M---------------------------------------  HCEBJ50XXprotein

24   FLSLDYIPQRKQEPIKDALMLFQRLQEKRMLEESNLSFLK  huFLICE(U58143)
41   FLCIGLVPNKKLEKSSSASDVFEHLLAEDLLSEEDPFFLA  HuMch4(U60519)
23   FLCRDVAIDVVPPNVRD---LLDILRERGKLSVGD---LA  HSALZ11Xprotein
2    ----------------------------------------  HCEBJ50XXprotein

64   ELLFRINRLDLLITYLNTRKEEME-RELQTPGRAQISAYR  huFLICE(U58143)
81   ELLYIIRQKKLL-QHLNCTKEEVE-RLL--PTRQRVSLFR  HuMch4(U60519)
57   ELLYRVRRFDLLKRILKMDRKAVETHLLRNP--HLVSDYR  HSALZ11Xprotein
2    ----------------------------------------  HCEBJ50XXprotein

103  VMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNLLDI  huFLICE(U58143)
117  NLLYELSEGIDSENLKDMIFLLKDSLPK----TEMTSLSF  HuMch4(U60519)
95   VLMAEIGEDLDKSDVSSLIFLMKDYMQRGKISKEKSFLDL  HSALZ11Xprotein
2    ---AEIGEDLDKSDVSSLIFLMKDYMQRGKISKEKSFLDL  HCEBJ50XXprotein

143  FIEMEKRVILGEGKLDILKRVCAQINKSLLKI-INDY---  huFLICE(U58143)
153  LAFLEKQGKIDEDNLTCLEDLCKTVVPKLLRN-IEKYK--  HuMch4(U60519)
135  VVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQS  HSALZ11Xprotein
39   VVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQS  HCEBJ50XXprotein
```

FIG.2A

```
179  ---------EEFSKERSSSLEGSPDEFS------NGEELC  huFLICE(U58143)
190  --------REKAIQIVTPPVDKEAESYQ------GEEEL-  HuMch4(U60519)
175  VQGAGTSYRNVLQAAIQKSLKDPSNNFRLHNGRSKEQRLK  HSALZ11Xprotein
79   VQGAGTSYRNVLQAAIQKSLKDPSNNFR------------  HCEBJ50XXprotein

204  GVMTISDSPREQDSESQT-------LDKVYQMKSKPRGYC  huFLICE(U58143)
215  ----VSQTDVKTFLEALP-------RAAVYRMNRNHRGLC  HuMch4(U60519)
215  EQLGAQQEPVKKSIQESEAFLPQSIPEERYKMKSKPLGIC  HSALZ11Xprotein
107  ------EEPVKKSIQESEAFLPQSIPEERYKMKSKPLGIC  HCEBJ50XXprotein

237  LIINNHNFAKAREKVPKLHSIRDRNGTHLDAGALTTTFEE  huFLICE(U58143)
244  VIVNNHSFT----------SLKDRQGTHKDAEILSHVFQW  HuMch4(U60519)
255  LIIDCIG--------------------NETELLRDTFTS  HSALZ11Xprotein
141  LIIDCIG--------------------NETELLRDTFTS  HCEBJ50XXprotein

277  LHFEIKPHDDCTVEQIYEILKIYQ-LMDHSNMDCFICCIL  huFLICE(U58143)
274  LGFTVHIHNNVTKVEMEMVLQKQKCNPAHADGDCFVFCIL  HuMch4(U60519)
274  LGYEVQKFLHLSMHGISQILGQFACMPEHRDYDSFVCVLV  HSALZ11Xprotein
160  LGYEVQKFLHLSMHGISQILGQFACMPEHRDYDSFVCVLV  HCEBJ50XXprotein

316  SHGDKGIIYGTDGQEP--PIYELTSQFTGLKCPSLAGKPK  huFLICE(U58143)
314  THGRFGAVYSSDEALI--PIREIMSHFTALQCPRLAEKPK  HuMch4(U60519)
314  SRGGSQSVYGVDQTHSGLPLHHIRRMFMGDSCPYLAGKPK  HSALZ11Xprotein
200  SRGGSQSVYGVDQTHSGLPLHHIRRMFMGDSCPYLAGKPK  HCEBJ50XXprotein
```

FIG.2B

```
354  VFFIQAC--QGDNYQKGIPVETDSEEQPYLEMDLSSPQTR  huFLICE(U58143)
352  LFFIQAC--QGEEIQPSVSIEADALNPEQAPTSLQDS---  HuMch4(U60519)
354  MFFIQNYVVSEGQLEDSSLLEVDGPAMKNVEFKAQKRGLC  HSALZ11Xprotein
240  MFFIQNYVVSDGQLEDSSLLEVDGPAMKNVEFKAQKRGLC  HCEBJ50XXprotein

392  YIPDEADFLLGMATVNNCVSYRNPAEGTWYIQSLCQSLRE  huFLICE(U58143)
387  -IPAEADFLLGLATVPGYVSFRHVEEGSWYIQSLCNHLKK  HuMch4(U60519)
394  TVHREADFFWSLCTADMSLLEQSHSSPSLYLQCLSQKLRQ  HSALZ11Xprotein
280  TVHREADFFWSLCTADMSLLEQSHSSPSLYLQCLSQKLRQ  HCEBJ50XXprotein

432  RCPRGDDILTILTEVNHYEVSNKDDKKNMGKQMPQPTFTL  huFLICE(U58143)
426  LVPRHEDILSILTAVN-DDVSRRVDKQGTKKQMPQPAFTL  HuMch4(U60519)
434  --ERKRPLLDLHIELNGYMYDWNSRVSAKEKYYVWLQHTL  HSALZ11Xprotein
320  --ERG---------TIPG-----SGITESKDMHFSSLGCIL  HCEBJ50XXprotein

471  RKKLVFP------SD    huFLICE(U58143)
465  RKKLVFPVPLDALSI    HuMch4(U60519)
472  RKKLIL-----SYT□    HSALZ11Xprotein
345  -----L-----DVL□    HCEBJ50XXprotein
```

FIG.2C

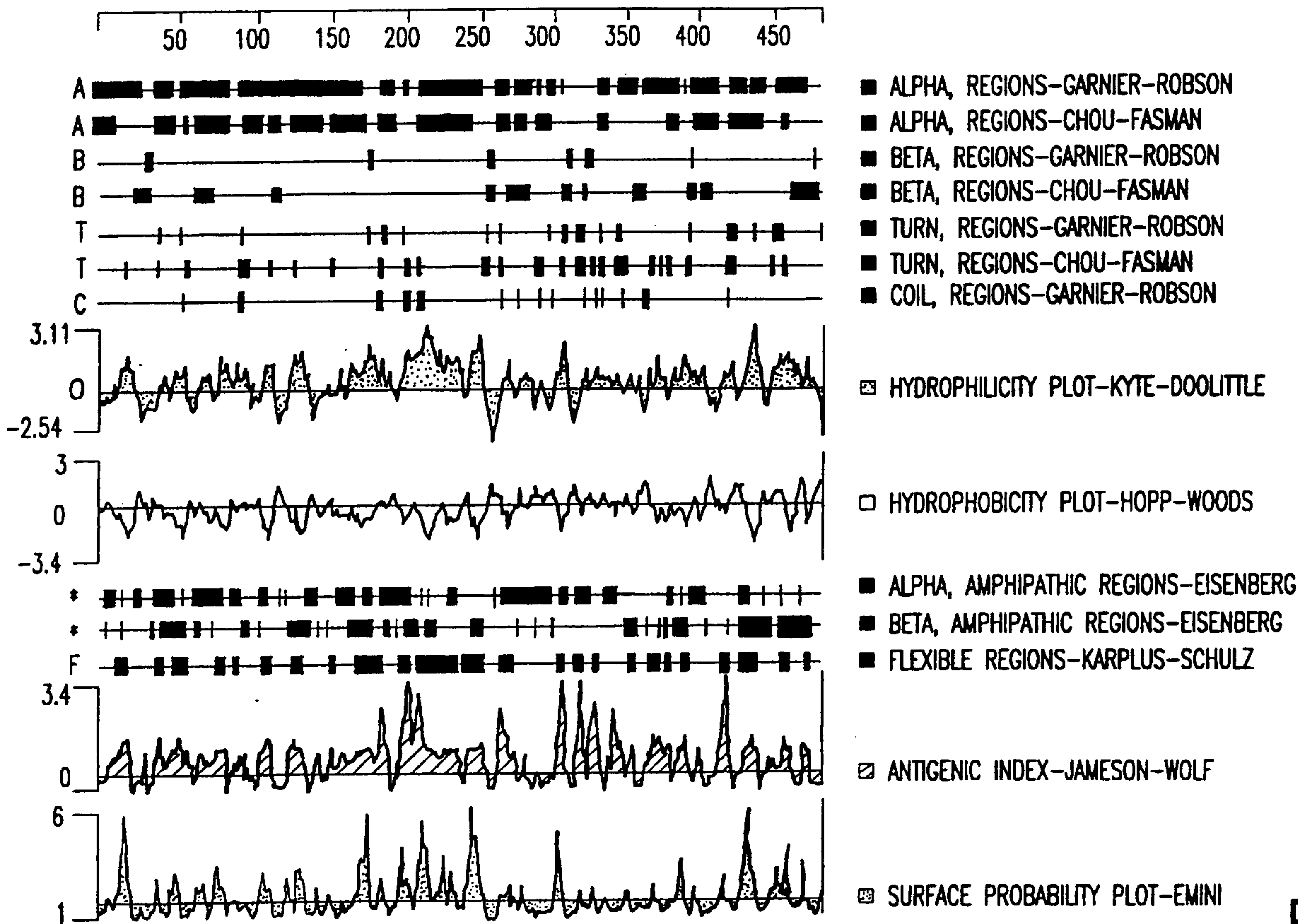
50
100
150
200
250
300
350
400
450
A
A
B
B
T
T
C
3.11
0
-2.54
3
0
-3.4
*
*
F
3.4
0
6
1
ALPHA, REGIONS-GARNIER-ROBSON
ALPHA, REGIONS-CHOU-FASMAN
BETA, REGIONS-GARNIER-ROBSON
BETA, REGIONS-CHOU-FASMAN
TURN, REGIONS-GARNIER-ROBSON
TURN, REGIONS-CHOU-FASMAN
COIL, REGIONS-GARNIER-ROBSON
HYDROPHILICITY PLOT-KYTE-DOOLITTLE
HYDROPHOBICITY PLOT-HOPP-WOODS
ALPHA, AMPHIPATHIC REGIONS-EISENBERG
BETA, AMPHIPATHIC REGIONS-EISENBERG
FLEXIBLE REGIONS-KARPLUS-SCHULZ
ANTIGENIC INDEX-JAMESON-WOLF
SURFACE PROBABILITY PLOT-EMINI

FIG.3

```
       10                             30                             50
GCGAGCTTGCAGCCTCACCGACGAGTCTCAACTAAAAGGGACTCCCGGAGCTAGGGGTGG

       70                             90                            110
GGACTCGGCCTCACACAGTGATTGCCGGCTATTGGACTTTTGTCCAGTGACAGCTGAGAC

      130                            150                            170
AACAAGGACCACGGGAGGAGGTGTAGGAGAGAAGCGCCGCGAACAGGCATCGCCCAGCAC

      190                            210                            230
CAAGTCCGCTTCCAGGCTTTCGGTTTCTTTGCCTCCATCTTGGGTGCGCCTTCCCGGCGT

      250                            270                            290
CTAGGGGAGCGAAGGCTGAGGTGGCAGCGGCAGGAGAGTCCGGCCGCGACAGGACGAGTG

      310                            330                            350
CTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCTCATTAATTTTCCTC
    M  A  E  I  G  E  D  L  D  K  S  D  V  S  S  L  I  F  L

      370                            390                            410
ATGAAGGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTTTCTTGGACCTTGTG
M  K  D  Y  M  G  R  G  K  I  S  K  E  K  S  F  L  D  L  V

      430                            450                            470
GTTGAGTTGGAGAAACTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGC
V  E  L  E  K  L  N  L  V  A  P  D  Q  L  D  L  L  E  K  C
      490                            510                            530
```

FIG.4A

CTAAAGAACATCCACAGAATAGACCTGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTT
L K N I H R I D L K T K I Q K Y K Q S V

550 570 590

CAAGGAGCAGGGACAAGTTACAGGAATGTTCTCCAAGCAGCAATCCAAAAGAGTCTCAAG
Q G A G T S Y R N V L Q A A I Q K S L K

610 630 650

GATCCTTCAAATAACTTCAGGGAAGAACCAGTGAAGAAATCCATTCAGGAATCAGAAGCT
D P S N N F R E E P V K K S I Q E S E A

670 690 710

TTTTTGCCTCAGAGCATACCTGAAGAGAGATACAAGATGAAGAGCAAGCCCCTAGGAATC
F L P Q S I P E E R Y K M K S K P L G I

730 750 770

TGCCTGATAATCGATTGCATTGGCAATGAGACAGAGCTTCTTCGAGACACCTTCACTTCC
C L I I D C I G N E T E L L R D T F T S

790 810 830

CTGGGCTATGAAGTCCAGAAATTCTTGCATCTCAGTATGCATGGTATATCCCAGATTCTT
L G Y E V Q K F L H L S M H G I S Q I L

850 870 890

GGCCAATTTGCCTGTATGCCCGAGCACCGAGACTACGACAGCTTTGTGTGTGTCCTGGTG
G Q F A C M P E H R D Y D S F V C V L V

910 930 950

AGCCGAGGAGGCTCCCAGAGTGTGTATGGTGTGGATCAGACTCACTCAGGGCTCCCCCTG
S R G G S Q S V Y G V D Q T H S G L P L

970 990 1010

CATCACATCAGGAGGATGTTCATGGGAGATTCATGCCCTTATCTAGCAGGGAAGCCAAAG
H H I R R M F M G D S C P Y L A G K P K

1030 1050 1070

ATGTTTTTTATTCAGAACTATGTGGTGTCAGACGGCCAGCTGGAGGACAGCAGCCTCTTG
M F F I Q N Y V V S D G Q L E D S S L L

1090 1110 1130

GAGGTGGATGGGCCAGCGATGAAGAATGTGGAATTCAAGGCTCAGAAGCGAGGGCTGTGC
E V D G P A M K N V E F K A Q K R G L C

1150 1170 1190

ACAGTTCACCGAGAAGCTGACTTCTTCTGGAGCCTGTGTACTGCGGACATGTCCCTGCTG
T V H R E A D F F W S L C T A D M S L L

1210 1230 1250

GAGCAGTCTCACAGCTCACCGTCCCTGTACCTGCAGTGCCTCTCCCAGAAACTGAGACAA
E Q S H S S P S L Y L Q C L S Q K L R Q

1270 1290 1310

GAAAGGGGGACAATTCCCGGAAGTGGAATTACAGAGTCAAAGGACATGCATTTTTCAAGC
E R G T I P G S G I T E S K D M H F S S

1330 1350 1370

CTCGGATGCATCTTACTAGATGTCCTATAGGATGGTCATATCAGCTTTATAGGAGAGTAG
L G C I L L D V L *

1390 1410 1430

CTGTGTCCCTGAATTCTCCCTGACACTGCATGCTCTTATATTTCCTCAAGTTTTGACAAT

1450 1470 1490

TTGATAGGTGAAAAGTGGTATCTGACTGTTCAGATCTGGAAGGCTTTGTTATATAAACAT

1510 1530 1550

TTTTTTAATGTTTATTGGCAAGAATACTTTTCTAAGAGAAACATCAGTGAGCTGGTTTCC

1570 1590 1610

ATTTAAGCTGAATGAAGCCACAATGTACCTCAAGTATAAGATTAACTGGCCTTTTTCAGT

1630 1650 1670

TGCACTCTAATTACAATTTAGAATGATGTTTCTGAGCCACCTGTCAAATGCATTCTGGGC

FIG.4B

1690 1710 1730
TGTACCTCTGCGTACCCCAGGAATAAATCTCATGGCCTTCTTTACCTGGCCTCCTTAGTG

1750 1770 1790
GTGGCCCAGCAGGAAGCGGGGGTTAGAGCAGGAGCCACTCAGCCTTCCAAGATAGATACT

1810 1830 1850
CCATGGGCCGGTGGTATTACTGGCCTTTTGAGCCCATCCCCATTTGCATAGATGATCCAC

1870 1890 1910
GTGGGTTATCATCTGGCTGGTATGTTCCCAGAGTGAAACTCAGCAGCCCCTTGAGGGAGG

1930 1950 1970
GGATGGTGGCCATCAGGCCAGAGTATTGCAAGTTAGTTTGGATCATTTGCTAAGCAGCTT

1990 2010 2030
GTGGTGCCTTCAGAAAGGAACAGTTTCAAAGAACTTTCACATCTGTTGGCTCATTTCGCC

2050 2070 2090
CTAATGACAGTCTTCTCTTTGATATTTGCATGGCATTAAATTTTGCCTTTCTTGTTTTCT

2110 2130 2150
CCAGAAAACGCCCACTCCTGGATCTTCACATTGAACTCAATGGCTACATGTATGATTGGA

2170 2190 2210
ACAGCAGAGTTTCTGCCAAGGAGAAATATTATGTCTGGCTGCAGCACACTCTGAGAAAGA

2230 2250 2270
AACTTATCTCTCCTACACATAAGAAACCAAAAGGCTGGGCGTAGTGGCTCGCACCTGTGA

2290 2310 2330
TCCCAGCACTTTGGGAGGCCGAGGAGGGCGGATCACTTCAGGTCGGGAGTTCGAGACCAG

2350 2370 2390
CCTGGCCAGCATGTGAACGCTGTCCCTAGTAGAAGTGCAAAAATTGGCTGGTGTGGGTGT

2410 2430 2450
GGGTACCCTGTATTCCCAGTTGCTTGGGGGGCTGAGGTGGGAGGATCTTTTGACCCCAGG

2470 2490 2510
AGTTCAGGGTCATAGCATGCTGTGATTGTGCCTACGAATAGCCACTGCATACCAACCTGG

2530 2550 2570
GCAATATAGCAAGATCCCATCTCTTTAAAAAAAAAAAAAAAAGGACAGGAACTATCTTAA

2590
AAAAAAAAAAAAAAAAA

FIG.4C

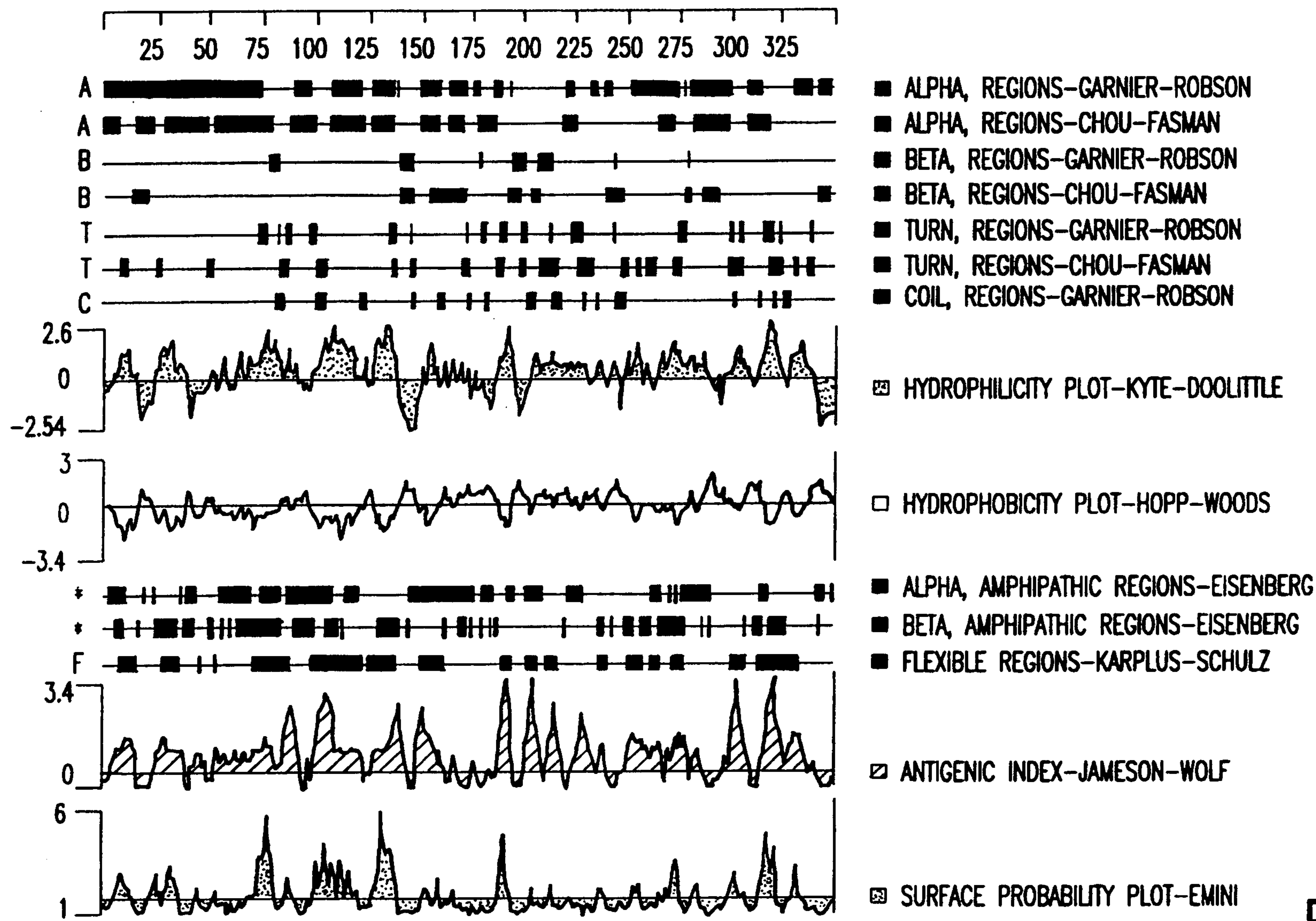
25
50
75
100
125
150
175
200
225
250
275
300
325
A
A
B
B
T
T
C
2.6
0
-2.54
3
0
-3.4
*
*
F
3.4
0
6
1
ALPHA, REGIONS-GARNIER-ROBSON
ALPHA, REGIONS-CHOU-FASMAN
BETA, REGIONS-GARNIER-ROBSON
BETA, REGIONS-CHOU-FASMAN
TURN, REGIONS-GARNIER-ROBSON
TURN, REGIONS-CHOU-FASMAN
COIL, REGIONS-GARNIER-ROBSON
HYDROPHILICITY PLOT-KYTE-DOOLITTLE
HYDROPHOBICITY PLOT-HOPP-WOODS
ALPHA, AMPHIPATHIC REGIONS-EISENBERG
BETA, AMPHIPATHIC REGIONS-EISENBERG
FLEXIBLE REGIONS-KARPLUS-SCHULZ
ANTIGENIC INDEX-JAMESON-WOLF
SURFACE PROBABILITY PLOT-EMINI

FIG.5

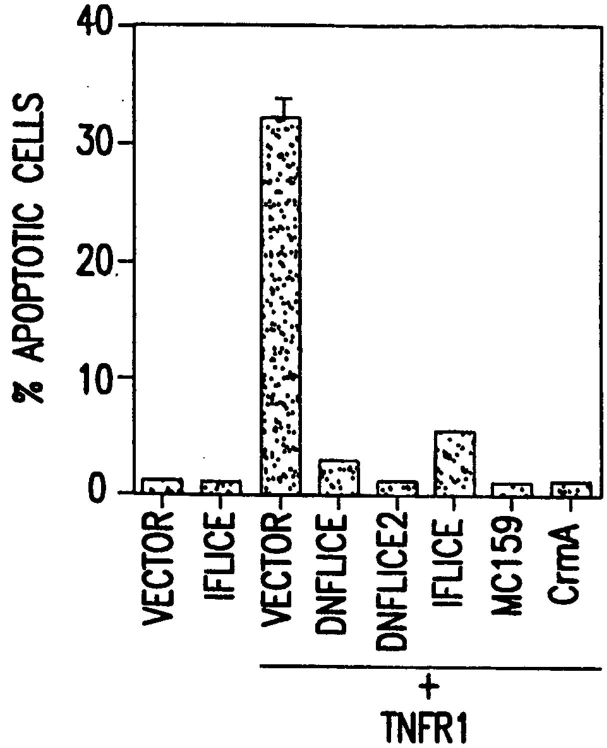
40
30
20
10
0
% APOPTOTIC CELLS
VECTOR
IFLICE
VECTOR
DNFLICE
DNFLICE2
IFLICE
MC159
CrmA
+
TNFR1

FIG.6A

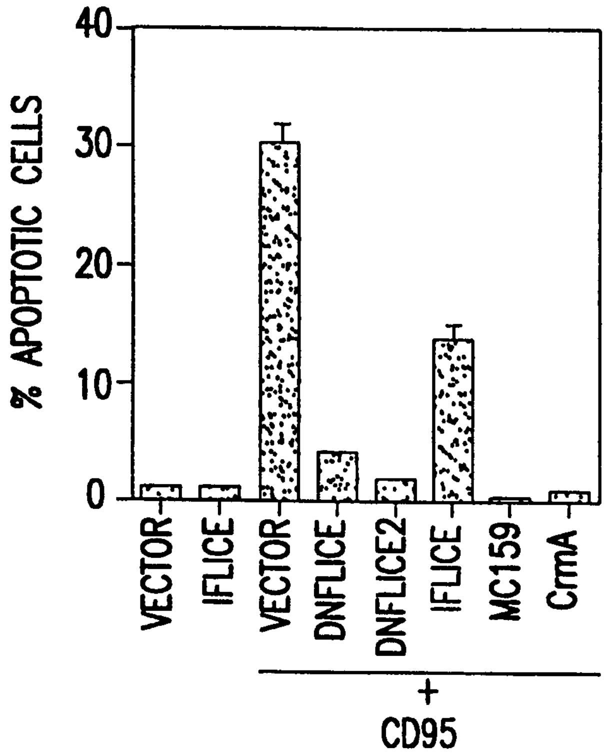
40
30
20
10
0
% APOPTOTIC CELLS
VECTOR
IFLICE
VECTOR
DNFLICE
DNFLICE2
IFLICE
MC159
CrmA
+
CD95

FIG.6B

reference number 1488.097PCU2 | PCT/US98/00969

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page __3__, line __28.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☒

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209038

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

DNA Plasmid 1631454

The applicants hereby request that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and is no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☒ This sheet was received by the International Bureau on: 20 MAY 1998
Authorized officer	Authorized officer JM

reference number 1488.097PC02	PCT/US98/00969

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page __4___, line _5_.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209041

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

DNA Plasmid 1789630

The applicants hereby request that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and is no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☑ This sheet was received by the International Bureau on: 20 MAY 1998
Authorized officer	Authorized officer JM

Applicant's or agent's file reference number 1488.097PC02 | International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 28.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209038

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

DNA Plasmid 1631454

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Sonya Barnes	
Authorized officer	Authorized officer

(DNA Plasmid 1631454)

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

DENMARK

The applicant hereby request that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

SWEDEN

The applicant hereby request that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

UNITED KINGDOM

The applicant hereby request that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

(DNA Plasmid 1631454)

NETHERLANDS

The applicant hereby request that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

reference number 1488.097PC02 | PCT/US 98/09... TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 5.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 15, 1997	ATCC 209041

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

DNA Plasmid 1789630

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Sonya D. Barnes	
Authorized officer	Authorized officer

(DNA Plasmid 1789630)

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

DENMARK

The applicant hereby request that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

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(DNA Plasmid 1789630)

NETHERLANDS

The applicant hereby request that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

Inte ...onal Application No
PCT/US 98/00969

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N9/64 A61K38/48 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "AC No. AA151642. Homo sapiens cDNA clone 588320" EMBL SEQUENCE DATABASE, 15 December 1996, HEIDELBERG, GERMANY, XP002063883 see the whole document ---	2,8-10, 12,14,15
X	TAKEDA J: "AC No. C05730. EST (expressed sequence tag). Homo sapiens." EMBL SEQUENCE DATABASE, 13 October 1996, HEIDELBERG, GERMANY, XP002063884 see the whole document ---	2,9,10, 12,14,15

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
29 April 1998	03.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHINNAIYAN A M AND DIXIT V M: "The cell-death machine" CURRENT BIOLOGY, vol. 6, no. 5, 1 May 1996, page 555-562 XP002063885 see page 558, paragraph 2 - page 559, paragraph 6; figure 3; table 1	
A	MUZIO M ET AL: "FLICE, A NOVEL FADD-HOMOLOGOUS ICE/CED-3-LIKE PROTEASE, IS RECRUITED TO THE CD95 (FAS/APO-1) DEATH-INDUCING SIGNALING COMPLEX" CELL, vol. 85, no. 6, 14 June 1996, pages 817-827, XP002037500 cited in the application see abstract; figures 2,3,7 see page 819 - page 822	
P,X	HU S ET AL: "I-FLICE, A NOVEL INHIBITOR OF TUMOR NECROSIS FACTOR RECEPTOR-1- AND CD-95-INDUCED APOPTOSIS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 28, 11 July 1997, pages 17255-17257, XP002051465 see the whole document	1-10, 12-15
P,X	IRMLER M ET AL.: "Inhibition of death receptor signals by cellular FLIP" NATURE, vol. 388, 10 July 1997, pages 190-195, XP002063886 see the whole document	1-10, 12-15

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/00969

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 11 is directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.